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Oral immunoprophylaxis using microencapsulated antigens as a disease-management strategy in farmed finfish populations

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For my grandmother, Prativa, who taught me to seek new perspectives, live life by my own rules and to treat every moment as an opportunity to learn something new

Declarations by the Author

Statement of Originality

This thesis contains no material that has been previously accepted for or submitted towards a degree or diploma by this University or any other institution except by way of background information and duly acknowledged in the thesis. The work presented in this thesis is original and my own, and to the best of my knowledge and belief contains no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University (AEC approval numbers: 11594 and A12285).

Signed: _____ Dated: 08/06/2015

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Statement of Co-Authorship

The following people and institutions contributed to the publication of the work undertaken as part of this thesis:

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Paper 1 (Located in Chapter 2)

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Conceived and designed the experiments	BG, BFN, ARB
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Analysed the data	BG
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List of Abbreviations

ag	attogram
ANOVA	analysis of variance
bp	base pair
BSA	bovine serum albumin
BSA	bovine serum albumin
d	day
df	degrees of freedom
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
FITC	Fluorescein Isothiocyanate
g	gram
g	gravity
GALT	gut-associated lymphoid tissue
GI	gastrointestinal
GiALT	gill-associated lymphoid tissue
h	hour
IgA	immunoglobulin A
IgD	immunoglobulin D
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IgT	immunoglobulin T
IP	intraperitoneal
kg	kilogram
KPBS	potassium phosphate buffered saline
L	litre
L-15	L15 Medium (Leibowitz) for cell culture
M	mole
MALT	mucosa-associated lymphoid tissue
mg	milligram
MHC	major histocompatibility complex
min	minute

mL	millilitre
mM	micromole
mm	millimetre
mRNA	messenger ribonucleic acid
n	number (of individuals/samples)
NALT	nasopharynx-associated lymphoid tissue
NCC	non-specific cytotoxic cells
nm	nanometer
OD	optical density
PAMP	pathogen associated molecular pattern
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PLGA	poly(lactic-co-glycolic acid)
PRR	pattern recognition receptor
qrtPCR	quantitative real-time polymerase chain reaction
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	room temperature
s	second
S.D.	standard deviation
S.E.	standard error
SALT	skin-associated lymphoid tissue
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
TBE	tris-buffered ethylenediaminetetraacetic acid
TSA	tryptone soy agar
TSB	tryptone soy broth
TYES	tryptone yeast extract salts
W	Watts
µL	microlitre
µm	micrometre

Note regarding thesis structure

Chapters 2 through to 4 of this thesis have independently been published (or submitted for publication) as journal articles, and presented here in their entirety. Consequently, there is some unavoidable overlap between material presented in these chapters, and in the introductory and concluding sections of this thesis. The first chapter of this thesis is written as a general introduction and review of relevant topics to provide necessary context and the experimental rationale for the research presented. As per the rules governing doctoral candidature in Australia, research representing the combined work of two or more concurrently enrolled PhD candidates cannot be presented in its entirety towards meeting the requirements for the degree. Accordingly, Chapter 6 presents only the results arising from experimental analysis performed by the candidate. However, salient outcomes from the combined work, presented in Appendix A, have been cited in order to provide a coherent discussion of experimental findings. Though included as discrete sections, the material in this thesis has been arranged to present a clear progression of techniques and knowledge culminating in the aims of this PhD project. The referencing style used by the journal Fish & Shellfish Immunology has been applied throughout and a combined bibliography for all the chapters is included at the end of this thesis; however, the orthography is consistent with the commonwealth countries of Britain.

Executive Summary

The increase in intensification of aquaculture production has been accompanied by an increase in impacts of pathogenic diseases. Large populations of stock maintained in close proximity have left much of the aquaculture industry susceptible to major economic losses caused by disease. As a consequence, and due to the variety of negative impacts associated with chemotherapeutics, development of immunoprophylaxis has received great impetus as a preventive disease management strategy for finfish. The most commonly used methods for fish immunoprophylaxis - injection and immersion - are generally associated with high procedural cost and access constraints. Oral immunoprophylaxis strategies for finfish, the main focus of this thesis, are able to effectively obviate these constraints and have therefore been proposed as an ideal approach for fish health management.

Due to performance inconsistencies linked to digestive degradation of orally administered antigens, oral immunoprophylaxis strategies have not been widely implemented in aquaculture. This thesis examined the feasibility of alginate microcapsules manufactured using a low impact technology and reagents to protect orally delivered immunogens for immunoprophylaxis of finfish. The microencapsulation method developed was found to be well suited for oral immunoprophylaxis of fish, as demonstrated by successful uptake of microcapsules and systemic distribution of contents *ex vivo* and *in vivo*, as well as the ability to affect controlled release of contents in target environmental conditions. The method also demonstrated no adverse impact on the integrity of the encapsulated substance, implying applicability to a broad range of immunoprophylactic materials.

The microencapsulation method was adapted for use with live microbial cells, and its viability as a disease management strategy was assessed against pathogenic disease of temperate and coldwater fish, bacterial coldwater disease (BCWD), which is caused by *Flavobacterium psychrophilum*. The protective efficacy of a putative probiotic *Enterobacter* species (C6-6) against BCWD was examined when administered as an alginate microencapsulated oral treatment. A similar trial was performed to test the effectiveness of an orally administered, alginate microencapsulated live-

attenuated vaccine (B17) against BCWD. In both trials, the modified method was successfully used to microencapsulate the live cell antigens while maintaining their viability. Though achieving significantly better fish survival than in untreated controls, oral administration of C6-6 was not as effective as intraperitoneal (IP) injection in protecting fish against BCWD. In contrast, orally administered B17 achieved similar serum antibody titres and survival as IP administered B17, with survival in both groups significantly better than untreated controls. An elevated challenge pressure made it difficult to draw clear conclusions regarding efficacy, though the similarities in treatment outcomes suggested that orally administered B17 could potentially approach the effectiveness of IP injected administration.

Yersinia ruckeri, the causative agent of yersiniosis in fish, is is a ubiquitous finfish pathogen affecting a broad range of species, and has been responsible for severe mortality in fish stocks globally. It is also capable of establishing and maintaining asymptomatic infections in apparently healthy fish, which act as reservoirs of infection within populations. A non-destructive technique for reliable detection of low levels of *Y. ruckeri* is necessary for effective management of the disease. A highly sensitive quantitative real-time PCR-based assay targeting the *Y. ruckeri* 16S-ribosomal gene was developed, capable of reliably detecting single-cell presence of the pathogen in spleen and faecal samples. The assay was able to detect *Y. ruckeri* in faecal samples at levels lower than previously possible, presenting the possibility of screening populations for asymptomatic infection without the need for invasive sampling.

Yersiniosis is conventionally managed by immersion immunisation in small fish and injected vaccines for larger fish. Large-scale *Y. ruckeri* infections have observed in fish smaller than typical minimum size immunised in the industry. Consequently, protecting fry at early developmental stages is important, and the effectiveness of an alginate-microencapsulated vaccine orally administered to first feeding fry was investigated. Significant protection following pathogenic challenge indicated considerable potential, though the treatments did not affect establishment rate of asymptomatic infection in survivors. The lack of typical adaptive immune responses made it

difficult to draw clear conclusions regarding the mechanisms responsible for the protection observed.

The work presented in this thesis establishes the feasibility of oral immunoprophylaxis for finfish. A versatile, low-impact alginate microencapsulation-based method for oral administration of a variety of immunogens is presented. Its potential as a health management strategy is demonstrated against known finfish diseases, though further optimisation of the approach will be greatly aided by an increased understanding of mucosal immune responses in finfish.

CHAPTER ONE

General Introduction

1.1. Teleost immunity

All multicellular organisms possess non-specific innate immune mechanisms that provide immediate protective responses against pathogenic assault. Among these organisms, only a subgroup of vertebrates possesses an additional specific – or acquired – immune response, with the most sophisticated acquired immune responses having been characterized in mammals (Table 1.1). While this dichotomy between innate and acquired immune systems is commonly referenced when discussing or characterizing immune responses, recent advances in our understanding of immunity have led to perception of nonspecific and specific immunity as being complementary parts of a unified host immune response against infection [1]. Teleosts occupy a key evolutionary position in the development of the innate and adaptive immune responses in being the earliest evolutionary class of vertebrates that are equipped with both innate and adaptive immunity.

Unlike most other vertebrates, teleosts become free-living organisms from an early stage in their life history. While they eventually develop a specific - or adaptive - immune system, nonspecific innate immunity is thought to protect them from pathogens through early stages of their development. The innate immune system also plays a key role in the development and activation of acquired immunity in teleosts. It is therefore fundamental to their ability to combat pathogens, particularly due to the relatively slow kinetics of specific immune responses and the limited availability of antibodies in the teleost repertoire in comparison with higher vertebrates [2]. Nonspecific immune responses do not need priming to interact with key components of pathogens, instead being triggered by germline-encoded pattern recognition receptors (PRRs) that recognise conserved pathogen-associated molecular patterns (PAMPs). The innate immune system includes physical barriers against pathogen infection, such as mucosal and epithelial tissues. Cell-mediated innate immune responses include phagocytic cells such as macrophages and neutrophils, as well as non-specific cytotoxic cells (NCC). Humoral innate immune factors such as lytic enzymes, anti-bacterial peptides, transferrins and complement proteins are capable of destroying or inhibiting growth of infectious microorganisms.

Table 1.1: Summary comparison of fundamental adaptive immune features in teleosts and mammals

Immunological feature	Teleosts	Mammals
Immunoglobulin	IgM, IgD and IgT (or IgZ)	IgM, IgG, IgA, IgD and IgE
Somatic hypermutation	+++	+++
Affinity maturation	Low	High
Memory responses	Weak	Strong
Antibody response	Slow	Fast
TCR, CD4, CD8	Yes	Yes
MHC class I and II	Yes	Yes
Spleen, thymus and bone marrow	Spleen and thymus but no bone marrow	Yes
Germinal centers and lymph nodes	No	Yes
Mucosa-associated lymphoid tissue	Yes	Yes

(adapted from [3, 4])

The teleost adaptive immune system consists of B lymphocytes (B cells), T lymphocytes (T cells), and antigen presenting cells. The lymphocytes express surface molecules that bind with receptors antigens (B cell receptors: BCR, and T cell receptors: TCR). Immune memory mechanisms and major histocompatibility (MH) products allow for clonal selection of a variety of B and T cells through somatic recombination to allow recognition of diverse pathogens [1, 5, 6]. Some T cells (cytotoxic T cells) express surface CD8 receptors and bind with MHC-I molecules, facilitating direct destruction of pathogen infected cells. Other T cells (T-helper cells) express surface CD4 receptors, and are involved in B cell activation and secretion of cytokines. B-cells are either activated by T-cell-secreted cytokines or by direct interaction with antigen, and subsequently differentiate to facilitate either pathogen destruction or formation of immunological memory [7]. The principal immunoglobulins involved in the teleost adaptive immune response belong to the IgM class, though two more immunoglobulins have recently been discovered: IgD [8] and IgT/IgZ [9, 10]. While the functionality of IgD is still unclear, IgT is associated with specialised mucosal immune responses [11, 12].

1.2. Lymphoid system in teleosts

The kidney, thymus and spleen are typically considered the most important lymphoid organs in teleosts. Recent research has identified the teleost liver as a possible immune organ, demonstrating response of intrahepatic lymphocytes to challenge [13], and the presence of T-cell associated interbranchial lymphoid tissue [14, 15]. However, further investigation may be required to identify the functional role of these organs.

Though haematopoietic progenitors may be observed in the anterior kidney early in the ontogeny of some species, the thymus matures into a lymphoid organ earliest among these organs in freshwater teleosts, with precursory development observed as early as 24 hours post-fertilization [16]. The anterior kidney, also known as the head kidney or pronephros, is the primary lymphoid organ in teleosts. It exhibits elevated haematopoietic function and is immunological analogous to that of bone marrow in higher vertebrates. It also functions as a secondary lymphoid organ comparable to mammalian lymph nodes, being involved in the induction and elaboration of immune responses, the clearance of soluble and particulate antigens from circulation, and the facilitation of immunological memory [17-19].

The thymus of teleost is located close to the gill cavity, can vary in morphology considerably between different species, and its appearance is influenced by life-history stage, hormonal cycles, and seasonal cycles [20]. Besides eosinophilic granulocytes and lymphocyte complexes with epithelial cells and thymic macrophages, the thymus is considered to be the main source of immunocompetent T-cells in teleosts. It is therefore crucial to the development of cell-mediated immune responses. The spleen is considered the principal peripheral lymphoid organ, composed of a fibrous capsule and small trabeculae that is usually extended into the parenchyma. The spleen represents a secondary lymphoid organ showing thrombopoietic and erythropoietic activity [21], and in adult teleosts it contains a large accumulation of B-cells.

1.3. Mucosa associated lymphoid tissues

Diffuse immune structures and mechanisms also occur at mucosal surfaces in teleosts, and their role in the teleost immune response has been gaining greater importance as more information regarding function and character of these tissues is discovered [22, 23]. Mucosal penetration represents the primary route of infection for most pathogens. Being the principal interface between the external environment and the internal environment of the organism, these mucosal surfaces also form the primary defence against infiltration of external pathogens and subsequent pathogenesis [24]. Considerable research has revealed specialized immunological adaptations at mucosal surfaces of vertebrates, including teleosts, that indicate the existence of mucosa-associated lymphoid tissues (MALT). Though the teleost MALT has not been studied in as great a depth as that of mammals, physiological, anatomical and morphological observations have provided sufficient evidence that the MALT in teleosts and mammals are quite different in their organization, and functionality of components is not directly translatable. Mucosal immunity is of particular importance for fish, as their aquatic environment potentially exposes them to continuous assault from pathogens, and consists not only of constitutive integumentary structures, but also cellular and humoral defences facilitated by specialised immunological adaptation in associated lymphoid tissues. Teleost mucus is known to contain lectins, complement proteins, pentraxins, lysozymes, antibacterial peptides and immunoglobulins; all of which contribute to a primary response against infiltrating pathogens [25-28]. These defensive adaptations are complemented by immune cells such as lymphocytes and macrophages [29, 30].

The teleost MALT can be differentiated into four major compartments, comprising the gut-associated lymphoid tissue (GALT, the skin associated lymphoid tissue (SALT) , the gill-associated lymphoid tissue (GiALT), and the nasopharynx-associated lymphoid tissue (NALT) recently identified in rainbow trout) [14, 31, 32].

1.4. Gut-associated lymphoid tissues (GALT)

The morphology and organization of the gastrointestinal (GI) tract exhibits broad variation across species of teleosts, reflecting differences in diets and feeding strategies. However, the intestinal tract in most teleosts can generally be differentiated into proximal and distal segments. Several researchers have shown that the hindgut or distal section of the intestine participates in engendering an immune response to enteric antigens. In mammals, the GALT consists of both scattered and organized lymphoid tissues. Specialised membranous cells (M-cells), present in the epithelium overlying the Peyer's Patches and in association with intestinal villi, take up and translocate these antigens to the mucosal lymphoid tissue, thereby initiating an immune response [31, 33]. While fish lack an organized GALT containing Peyer's Patches, the distal section of the gastrointestinal tract in teleosts is capable of antigen uptake and translocation of these antigens to immune cells in the lamina propria that include macrophages, granulocytes, lymphocytes and plasma cells, and subsequently, via an intraepithelial layer populated by T cells (and some B cells) to systemic lymphoid organs [34-39] (Fig. 1.1). Certain epithelial cells associated with the GALT of salmonids display morphological similarities with mammalian M cells, which may facilitate this phenomenon by sampling antigens from the enteric lumen [40]. Similar uptake of intestinal antigens has been observed in other species as well, though the cells involved in the process were different [41]. While our understanding of the varying mechanisms involved is limited, this phenomenon of intestinal antigen uptake in teleosts has been widely accepted, with further confirmation in more recent studies [31, 42, 43]. The recent discovery of GALT-associated IgT, a novel immunoglobulin specialized in mucosal immunity, in rainbow trout indicates the possibility of a mucosal antibody response engendered in the GALT [11, 12].

1.4.1. The gut microbiome

There has been growing interest in the immunological role played by commensal microorganisms associated with the teleost gut. Composition of the gut microbiome can considerably impact the development of the GALT [44]. The microbiota colonizing the gut in zebrafish was found to

influence the development of innate immunity and epithelial barrier function [45]. Considerable research has been focused on the development of probiotics and prebiotics, in an effort to stimulate immunity against various pathogens through the gut microbiome [46-49]. While the natural composition of bacterial populations within the teleost gut are likely even more variable than the organization of the GALT, being influenced by myriad factors, several studies have found encouraging evidence that successful stimulation of immune responses against pathogens may be possible through modification of the teleost gut microbiome [23, 50].

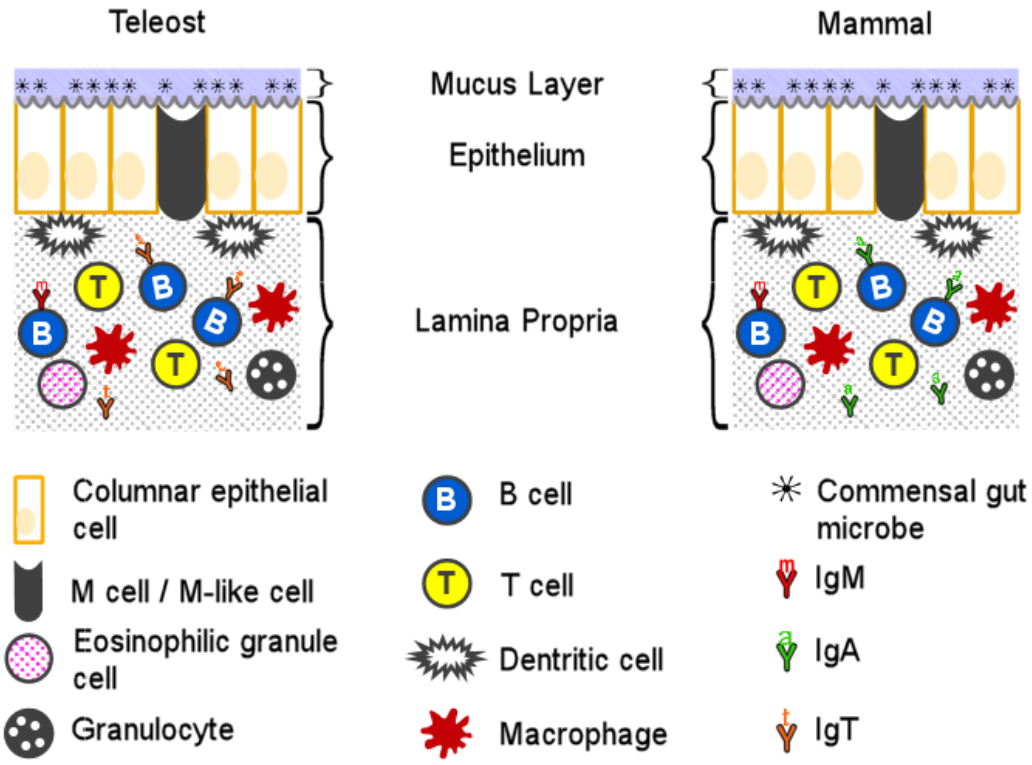

Figure 1.1: Schematic comparative showing immunological organisation of teleost GALT and comparable mammalian mucosa (Type I mucosa) (Modified from [23]).

1.5. Immunoprophylaxis in finfish aquaculture

Pathogenic diseases pose a major challenge to the viability of all livestock industries, including the culture of teleost species, and impacts were traditionally mitigated through the administration of chemotherapeutic compounds. The presence of the teleosts adaptive immune system presents the possibility of preventing disease through immunoprophylaxis, first validated successfully against furunculosis by Duff [51]. Development and implementation of preventive strategies for

management of disease in finfish culture through immunoprophylaxis, particularly through the use of vaccines, has received great impetus given the potential risks associated with the use of chemicals including antibiotics and the difficulties with medicating diseased fish [52, 53]. Immunoprophylaxis of fish using vaccines has been effective in managing diseases in aquaculture, and the impact of this strategy is reflected in the drastic reduction in antimicrobial administration possible in spite of increasing production, as illustrated by the Norwegian aquaculture industry [54, 55] (Fig. 1.2A). While early vaccines simply consisted of inactivated pathogens, greater understanding of the antigenicity of some pathogens has allowed the development of subunit vaccines, as well as recombinant protein and DNA vaccines, which have the potential to maximize immunogenicity [56-58].

Commercially, the most widely used methods for immunoprophylaxis of finfish are injection and immersion. Vaccines administered by injection to teleost species, either intraperitoneally (IP) or intramuscularly (IM), have proved relatively more effective method in protecting fish stocks from pathogens [59-62] (Fig. 1.2B). Injected vaccines typically achieve high levels of protection, which may be further enhanced by the simultaneous application of adjuvants. This can be accomplished without much difficulty when using injection administration. Injection vaccination can prove very economical in contrast to immersion vaccination when used for large fish [63], but typically cannot be used effectively in fish smaller than 20 g [64]. However, this strategy is typically very labour intensive, requiring specialized equipment and personnel training. Injection of vaccines requires that fish be anaesthetised and individual fish handled during the process.

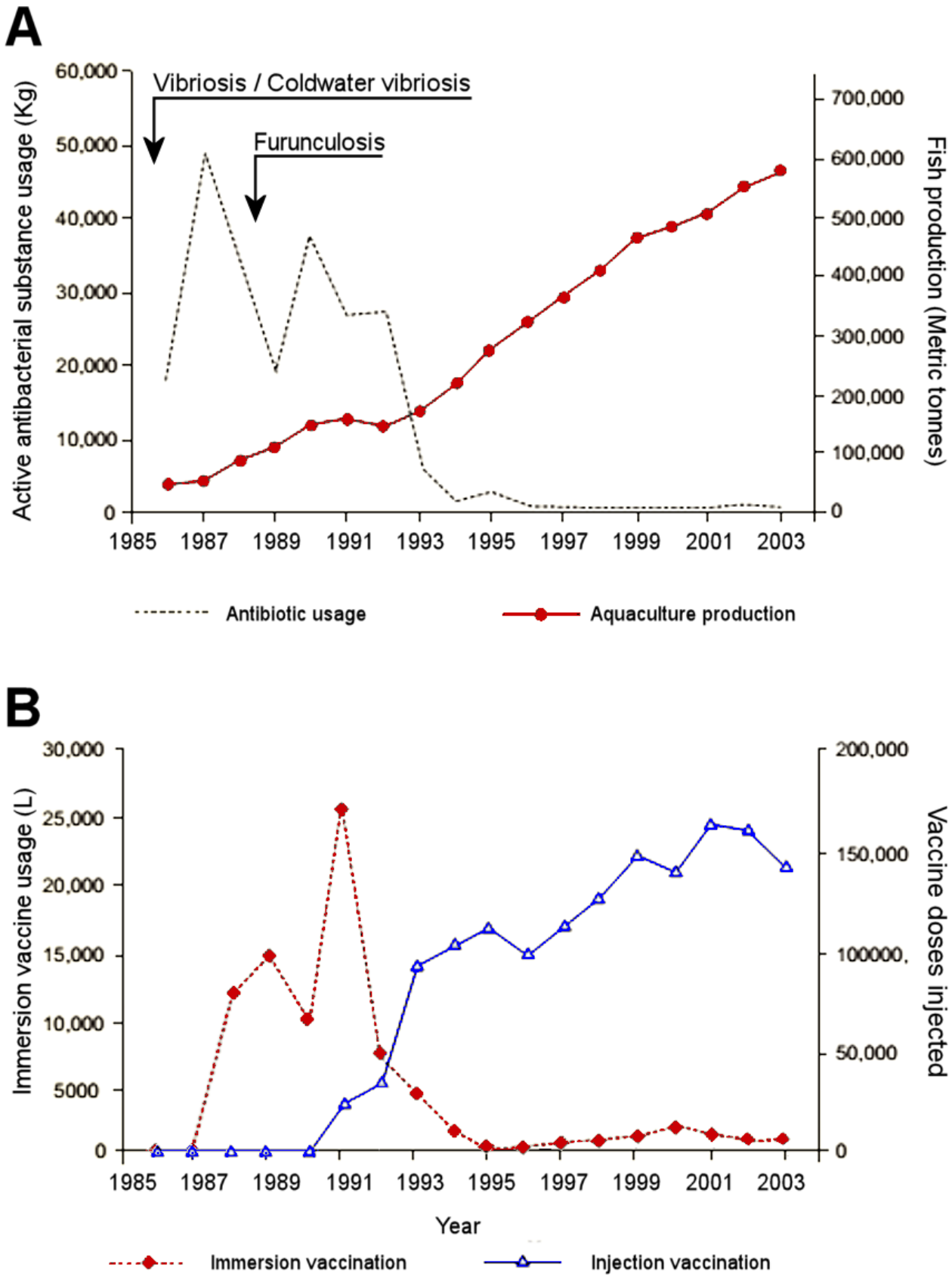

Figure 1.2: Introduction and adoption of vaccines compared with usage of antibiotics (A); and the usage of different vaccination approaches (B) in the Norwegian aquaculture industry [54, 65, 66].

This causes high levels of stress to stock and can result increased susceptibility to other diseases and in retardation of growth rate over extended periods [67-69]. Studies have associated injection vaccination, and specifically the use of adjuvants like mineral oil, with the formation of lesions that can negatively impact marketability [60, 70, 71]. While the introduction of injection machines have served to reduce the labour-intensive nature of the method, the impacts to stock remain [53, 64]. Immersion vaccination, typically for short periods in concentrated vaccine solution, has achieved substantial protection of finfish stocks against some diseases, and is widely used in aquaculture [54, 72]. It allows in-situ vaccination of stock, and considerably reduces stress compared to injection. However, the method is labour intensive, and is extremely uneconomical for large fish due to the volume of vaccine required [54, 64]. As a result of these drawbacks, adoption of these vaccination methods has been restricted mostly to the culture of high-value stock.

Oral administration has been proposed as an ideal alternative method for immunoprophylaxis of finfish, obviating the limitations associated with injection and immersion vaccination [73]. Oral immunoprophylaxis of fish does not result in stress to stock, can be used for fish of any size and is suitable for intensive and extensive farming practices. This approach does not require any specialised training, infrastructure, or disruption of production processes, thus significantly decreasing associated costs [74, 75]. Oral immunoprophylaxis also presents a strategy for targeting mucosal immunity in finfish directly, while also engendering a sustained immune response through intestinal uptake and eventual systemic distribution of antigenic material. Our present understanding of the antigen-sampling abilities of the teleost GALT supports the principles underpinning oral administration of immunoprophylactics to finfish stocks [34-39, 76, 77]. However, trials attempting to protect fish against pathogens by oral administration of antigens have yielded inconsistent results, generally achieving lower levels of protection than injection or immersion immunisation [53, 78-80]. While the method holds great potential as a convenient delivery strategy of immunoprophylactics to finfish, the inconsistency in performance must be addressed before it can be adopted on a broader scale.

Digestive degradation of antigenic matter in the anterior section of the GI tract is considered the major cause of inconsistency in efficacy of orally delivered fish immunoprophylaxis. Researchers have suggested that protecting antigens until uptake in the distal intestine would result in greater immunogenicity [31, 37, 41, 81, 82]. This has been confirmed by successful outcomes from studies employing some form of antigen protection [83] [84].

Due to easy availability and biocompatibility, encapsulation in biodegradable polymers have attracted considerable research interest as a means for protecting orally administered antigens [85]. Besides protection of immunogenicity, biopolymers also present a number of potential benefits, including controlled release of contents over time and the possibility of intrinsic adjuvant properties such as immunostimulation and mucoadhesion [86, 87].

1.6. Aims and outline of thesis

Orally administered immunoprophylaxis represents an appealing strategy for the management of disease in farmed fish stocks. The potential applicability of the method is strongly supported by considerable research on the uptake ability of the teleost intestine, and the immunological role of the GALT [11, 22, 30, 31, 33, 36-38, 40]. However, protection of orally administered antigenic material appears to be of primary importance in achieving wide-scale applicability of the strategy [72]. With the range of possible immunoprophylactic benefits available through the use of biopolymers, polymeric microencapsulation of antigens prior to administration could considerably enhance efficacy of oral immunoprophylaxis of finfish [88, 89]. Further, in having no intrinsic limitations on the size of fish the method can be applied to, an adequately protected antigen could potentially be administered orally to protect fish at a range of sizes [72]. Similarly, oral immunoprophylaxis could be used to effectively prolong protection against a pathogen as antigen administration would not necessitate any disruption of the production cycle or cause stress to fish [90].

The aims of this research project were:

- to develop effective protection from digestive degradation for a range of antigenic material for oral immunoprophylaxis of finfish, and
- to evaluate effectiveness of this orally administered approach in protecting fish against pathogenic challenge using different immunoprophylactics

In Chapter 2, a method for polymeric microencapsulation of a soluble antigen was developed and assessed in the context of oral immunoprophylaxis for fish. Uptake and translocation of microencapsulated antigen in the teleost intestine was examined *ex vivo* and *in vivo*.

In Chapter 3, the previously developed microencapsulation method was applied with modifications to a putative probiotic *Enterobacter* sp. (C6-6) inhibitory to *Flavobacterium psychrophilum*, isolated from the gastrointestinal tract of rainbow trout. The ability to successfully microencapsulate C6-6 cells while maintaining viability was examined. Differences in protective efficacy of C6-6 against *F. psychrophilum* challenge when administered to rainbow trout orally or via IP injection, with or without microencapsulation, were assessed.

In Chapter 4, a recently developed live-attenuated vaccine against *F. psychrophilum* used to immunise rainbow trout by oral or IP administration, both with and without microencapsulation. Relative efficacy of the different immunisation administration methods was assessed through pathogenic *F. psychrophilum* challenge.

In Chapter 5, a quantitative real-time PCR-based method for the detection of minute levels of *Yersinia ruckeri* was developed. The method was used to detect and quantify low levels of *Y. ruckeri* in different organic matrices, and its applicability as a non-lethal technique for detection of low infection levels in Atlantic salmon populations was assessed.

In Chapter 6, a microencapsulated vaccine against *Y. ruckeri* was developed, and used to immunise first-feeding fry. Protective efficacy of the vaccine was assessed through pathogenic *Y. ruckeri*

challenge. Challenge survivors were examined for asymptomatic *Y. ruckeri* infection using the previously developed quantitative real-time PCR-based method.

CHAPTER TWO

Alginate microencapsulation for oral immunisation of finfish: release characteristics, *ex vivo* intestinal uptake and *in vivo* administration in Atlantic salmon, *Salmo salar* L.

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2.1. Abstract

This study examined the feasibility of alginate microcapsules manufactured using a low impact technology and reagents to protect orally delivered immunogens for use as immunoprophylactics for fish. Physical characteristics and protein release kinetics of the microcapsules were examined at different pH and temperature levels using a microencapsulated model protein, BSA. Impact of the microencapsulation process on contents was determined by analysing change in bioactivity of microencapsulated lysozyme. Feasibility of the method for oral immunoprophylaxis of finfish was assessed using FITC-labelled microcapsules. These were applied to distal-intestinal explants of Atlantic salmon (*Salmo salar*) to investigate uptake *ex vivo*. Systemic distribution of microcapsules was investigated by oral administration of FITC-labelled microcapsules to Atlantic salmon fry by incorporating into feed. The microcapsules produced were structurally robust and retained surface integrity, with a modal size distribution of 250-750 nm and a tendency to aggregate. Entrapment efficiency of microencapsulation was 51.2% for BSA and 43.2% in the case of lysozyme. Microcapsules demonstrated controlled release of protein, which increased with increasing pH or temperature, and the process had no significant negative effect on bioactivity of lysozyme. Uptake of fluorescent-labelled microcapsules was clearly demonstrated by intestinal explants over a 24 h period. Evidence of microcapsules was found in the intestine, spleen, kidney and liver of fry following oral administration. Amenability of the microcapsules to intestinal uptake and distribution reinforced the strong potential for use of this microencapsulation method in oral immunoprophylaxis of finfish using sensitive immunogenic substances.

2.2. Introduction

Development and implementation of preventive strategies for management of disease in aquaculture through immunoprophylaxis, particularly through the use of vaccines, has received great impetus given the potential risks associated with the use of chemicals including antibiotics and the difficulties with medicating diseased fish [52, 53].

Immunoprophylaxis of fish using vaccines has been effective in managing diseases in aquaculture [54, 55]. The most commonly used methods for vaccine administration – injection and immersion – are generally limited to high-value segments of the aquaculture industry. This is mainly due to procedural cost and access constraints, to which oral immunisation has been proposed as an ideal alternative [73]. Oral immunoprophylaxis of fish causes no extra stress to stock, is appropriate for fish of any size and suitable for intensive and extensive farming practices. This approach does not require any specialised training, infrastructure, or disruption of production processes, thus significantly decreasing associated costs [74, 75]. In mammals, M-cells in the Peyer's patches and other areas of gut associated lymphoid tissue (GALT) play an important part in the uptake and presentation of orally introduced antigens to the immune system [76, 77]. Specialised cells (M-cells), present in the epithelium overlying the Peyer's patches, take up and translocate these antigens to the mucosal lymphoid tissue, thereby initiating an immune response. While fish lack Peyer's patches, early work provided evidence that the distal section of the gastrointestinal tract in teleosts was capable of antigen uptake and translocation of these antigens to macrophages and lymphocytes in the lamina propria and systemic lymphoid organs [34-39]. This phenomenon has been widely accepted, with further confirmation in more recent studies [31, 42, 43], and together with findings from more extensive investigation of intestinal immune functions [31], supports the potential for oral immunoprophylaxis of fish. Despite evidence of antigen uptake and immune interaction the results of oral delivery trials have been inconsistent, and were less efficacious than injection or immersion immunisation in protecting fish against infectious diseases [53, 78-80].

The inconsistent efficacy of orally delivered fish immunoprophylaxis has been attributed mainly to the digestive degradation of antigenic matter in the anterior section of the gut, implying that adequate protection of antigen integrity until uptake in the distal intestine would result in a greater immune response [31, 37, 41, 81, 82]. Studies employing antigen protection, such as enteric coating [83] and bio-encapsulation [84] in oral fish vaccines have further confirmed this opinion.

There has been increasing research interest in the protection of antigens for oral immunoprophylaxis of fish using biodegradable polymers due to the easy availability and biocompatibility of these polymers [85]. Polymer microencapsulation is also capable of controlled release of antigenic material, potentially minimising or even removing the current need for repeated administration of some vaccines [74, 91, 92]. Various methods are commonly used for microencapsulation of antigens, such as emulsification-solvent evaporation [93], coacervation [94], and spray drying [95]. Since the primary objective of microencapsulation is protection, the impact of the process on viability of the immunogenic material is a critical consideration. The three dimensional structure of proteins and peptides, which form the functional constituents of most immunoprophylactic applications, is essential to their bioactivity and can be easily disrupted by exposure to harsh environmental conditions that may be involved in some microencapsulation techniques [96, 97]. The emulsification-solvent extraction method, one of the most popular techniques, consists of preparing an emulsion with immiscible internal and external phases chosen according to the nature of the polymer used for the encapsulation and the material to be encapsulated, and evaporating or extracting the solvent by some other method with the subsequent formation of microcapsules [93]. The mild processing conditions employed by this method, combined with a careful choice of reagents, aim to minimise the impact of microencapsulation on bioactivity of encapsulated substances. Alginate is one of the most widely used polymers for microencapsulation of antigens, and its resistance to proteolysis, biodegradability, high biocompatibility, mucoadhesive properties, ease of availability and relatively low cost are very attractive when compared to other available polymers [86, 87].

The aim of this study was to assess the characteristics of alginate microcapsules produced using an emulsion-based method, combined with internal-gelation to increase structural integrity, for use as an oral delivery system of immunoprophylactics for fish. Structural properties and release characteristics were examined by using a model protein, bovine serum albumin (BSA), microencapsulated in alginate. Uptake of these microcapsules from the gastrointestinal (GI) tract of

Atlantic salmon (*Salmo salar*), and subsequent systemic distribution, was studied using fluorescent-labelled alginate in the manufacturing process. The impact of the selected microencapsulation method on the integrity of an encapsulated substance was assessed by microencapsulating lysozyme and assessing retained bioactivity.

2.3. Materials and methods

2.3.1. Microencapsulation

Bovine Serum albumin (BSA; Amresco , Solon OH, USA) was encapsulated in alginate crosslinked with calcium using an emulsification/internal-gelation based method adapted from Zheng *et al.* [98].

2.3.1.1. BSA-loaded Alginate microcapsule manufacture

Techniques were modified with the aim of achieving microcapsules smaller than 5 µm, and accordingly 600 mg medium viscosity sodium alginate salt (Sigma-Aldrich, St. Louis MO, USA) was dissolved in 20 mL distilled water under constant magnetic stirring at high speed. To this solution, 60 mg BSA dissolved in 0.5 mL distilled water was added and stirring was continued for 10 min to ensure BSA solution was homogenously dispersed through the alginate solution to form an aqueous phase. To form the oil phase, 3 mL Span-80 (Sigma-Aldrich) was thoroughly dispersed in 37 mL octane (Sigma-Aldrich). The aqueous phase was then gradually introduced into the oil phase while stirring at high speed using a handheld pitched-blade homogeniser. Stirring was continued for 3 min to achieve a water-in-oil emulsion. The emulsion was stirred for a further 5 min after addition of 3 mL Tween-80 (Sigma-Aldrich). Stirring continued over 25 min, following the drop-wise addition of 25 mL 8% aqueous solution of calcium chloride (Sigma-Aldrich), to crosslink microcapsules. To break the emulsion 55 mL isopropyl alcohol (Sigma-Aldrich) was added, and the final mixture was stirred for 25 min using a magnetic stirrer to harden the microcapsules. BSA-loaded microcapsules were recovered by centrifuging the mixture at 250x*g* for 10 min to separate out larger microcapsules, and then for 10 min at 14500x*g* to recover smaller microcapsules.

Recovered capsules were washed twice in distilled water and then lyophilised and stored at 4°C until further analysis.

2.3.1.2. Microcapsule characterisation

BSA-loaded microcapsules in aqueous suspension were applied to 12.6 mm diameter aluminium SEM mounts (ProSciTech, QLD, Australia) during lyophilisation. Lyophilised samples were then sputter coated with platinum and examined by Scanning Electron Microscopy (SEM) using the Electron Microscopy and X-Ray Microanalysis services (SU-70 field emission scanning electron microscope, Hitachi, Japan) of the Central Science Laboratory, University of Tasmania. 827 microcapsules randomly selected from scanning electron micrographs were examined for size and morphology. Structural stability was assessed by observing microcapsules for any deformation occurring over 60 s of elevated electron bombardment. Images were manually inspected to ensure they represented single unbroken microcapsules, and then analysed using ImageJ, a public domain Java image processing program [99], to determine size distribution. For each particle analysed, the Feret's diameter (largest axis length) was used as a measurement of size, and particles were accordingly grouped into 250 nm size-classes.

2.3.2. Protein encapsulation assessment

Protein quantitation was achieved using the micro-assay procedure for the Pierce Coomassie Protein Assay Reagent kit (Pierce Biotechnology, Thermo Scientific, Rockford IL, USA), as per supplied instructions.

Briefly, the supplied BSA standard (2 mg mL^{-1}) was reacted at various dilutions, at 150 μL with an equal volume of the Coomassie-dye reagent to establish a standard curve of absorbance at 595 nm, obtained using a plate reader (Tecan Thermo-Spectra Rainbow, Austria). A 150 μL volume of each sample was combined with an equal volume of reagent, and absorbance at 595 nm was compared with the standard curve to quantify protein content.

2.3.2.1. Loading efficiency

During the microcapsule manufacturing process, supernatant remaining after microcapsule recovery (by centrifuging) was retained and analysed for protein content. The loading efficiency of the microencapsulation process was calculated as:

Loading Efficiency (%) = $(C_I - C_F)/C_I \times 100$,

where C_I is the initial BSA concentration in solution before encapsulation and C_F is the final BSA concentration as observed. The supernatant was diluted in an equal volume of distilled water before samples were collected and analysed in triplicate. This was done to overcome the limitations of the assay's range of sensitivity (1-25 µg mL^{-1}).

2.3.2.2. Entrapment efficiency

Three 60 mg samples of microcapsules were suspended in 1.0 mL distilled water and a tissue homogeniser was used for 1 minute at 12000 rpm, followed by ultrasonication at 20W for a total of 5 min (30 s on: 30 s off) to disintegrate particles. The resulting suspensions were centrifuged at 16500x*g* for 15 min to remove microcapsule fragments, and the supernatant was analysed for protein content. The entrapment efficiency was expressed as the ratio of protein weight in solution to weight of polymer + expected weight of protein (as estimated by loading efficiency).

2.3.3. Protein release rate

Rate of release of BSA from alginate microcapsules was evaluated for different pH values and different temperatures. Samples were collected over 24 hours, at time points selected to simulate release behavior immediately post-prandium, as well as over sustained residence in the GI tract. Protein quantitation was carried out using the Pierce Coomassie Protein Assay Reagent kit, as described earlier.

2.3.3.1. pH-dependent release rates

Samples were prepared in triplicate for each planned measurement. Microcapsules were suspended in tubes containing 0.5 mL distilled water (1.5% w/w), to which either acetic acid or sodium

hydroxide had been previously added as necessary to achieve pH levels of 4.5, 7 and 9. Samples were collected at 1, 3, 6 and 24 h after suspension of microcapsules, and centrifuged at 14000x*g* to pellet the microcapsules. The supernatant was then collected and stored at 4°C until analysis of protein content to determine protein release from microcapsules.

2.3.3.2. Temperature-dependent release rates

Samples were prepared in triplicate for each planned measurement. Microcapsules were suspended in tubes containing 0.5 mL distilled water (1.5% w/w), which were incubated at 4, 12 or 37°C. Samples were collected at 1, 3, 6 and 24 h after suspension of microcapsules, and centrifuged at 14000x*g* to pellet the microcapsules. The supernatant was then collected and stored at 4°C until analysis of protein content to determine protein release from microcapsules.

2.3.4. Effect of microencapsulation on bioactivity

Encapsulation efficiency of lysozyme in alginate was used to determine the mean concentration of lysozyme expected in each sample. Lytic activity of known concentrations of lysozyme, and of samples, was then compared to establish any change in bioactivity of lysozyme as a result of microencapsulation.

2.3.4.1. Lysozyme bioactivity assay

Alginate microcapsules containing chicken egg white lysozyme were produced using a process identical to that used for production of BSA-alginate microcapsules as described, with the substitution of BSA with 150 mg chicken egg white lysozyme (49900 units mg^{-1}, Sigma-Aldrich). Encapsulation efficiency was determined using a Pierce-Coomassie colourimetric assay to analyse residual protein in supernatant as described for BSA.

Six 1.5 mL vials were prepared, each with 1.0 mL distilled water, and 12.5 mg lyophilised lysozyme-alginate microcapsules were added to each vial. The lysozyme was eluted out of the microcapsules by incubating the vials at 37˚C for 18 h, and then centrifuging at 14000x*g* for 5 min to pellet out microcapsules. Supernatant was collected from each vial, and diluted in 9 parts distilled

water. Controls were prepared by adding blank (alginate only) microcapsules to six 1.5 mL vials and processing under identical conditions.

A turbidimetric lysozyme activity assay was adapted from Parry *et al.* [100]. Briefly, 175 µl of *Micrococcus luteus* solution prepared in 0.1 M phosphate/citrate buffer was added to serially varying concentrations of chicken egg white lysozyme, and read in a plate reader (Tecan Thermo-Spectra Rainbow, Austria) at 450 nm over 15 min at 15 s intervals to establish a standard curve of change in absorbance over time. The procedure was repeated using experimental samples and controls in place of prepared lysozyme, and any changes in absorbance were compared to the standard curve to determine comparative activity of lysozyme eluted from microcapsules.

2.3.5. Uptake by intestinal epithelium *ex vivo*, and *in vivo* systemic distribution of orally administered microcapsules

Transport across epithelial membrane of intestinal explants was assessed using fluorescent-tagged microcapsules and fluorescent microscopy. BSA was labelled with FITC (Fluorescein Isothiocyanate; FITC-BSA) as in Hungerford *et al.* [101], and microencapsulated in FITC-labelled alginate microcapsules, which were produced as described below. All procedures on fish were performed in accordance with approved animal handling guidelines (University of Tasmania Animal Ethics Committee approval Ref: 11594).

2.3.5.1. FITC-labelled alginate microcapsule manufacture

Fluorescein isothiocyanate (FITC; Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) to obtain a 1 mg mL^{-1} solution. The FITC-DMSO solution was added to sodium alginate (3% aqueous) solution (3:1 v/v) that had been adjusted to pH 9 and following continuous stirring for 1 h at 40°C, ethanolamine was added to the mixture (15% v/v) to stop the reaction. FITC-labelled alginate solution was dialysed against 500 mL of distilled water for 18 h at 8°C to remove any uncoupled FITC. To 29.5 mL of this solution, 120 mg FITC-BSA (dissolved in 0.5 mL distilled water) was added and stirring was continued for 10 minutes to ensure BSA solution was homogenously dispersed through alginate solution to form the aqueous phase. This aqueous phase

was used to manufacture microcapsules using an emulsion based method as described in section 2.1.1.

2.3.5.2. *Ex vivo* Intestinal uptake assay

Sections of intestine measuring 5 cm in length were removed from five Atlantic salmon (*Salmo salar*), weighing approximately 750 g each, that had been lethally anaesthetised using clove oil (200 mg L^{-1}) and prepared for *ex vivo* analysis using techniques adapted from a previously published method [102]. Intestinal sections were gently sliced open along the longitudinal axis, and laid flat (serosal side down) on a chilled cutting surface. Each section was cut into pieces of approximately 4 x 4 mm. Individual pieces were placed in separate wells of a 96-well microplate containing Leibowitz 15 (L15) medium and incubated at 15°C until required.

Controls were established by either adding 150 µL unencapsulated FITC-PBS solution, or only PBS, to intestinal explants. Experimental treatment consisted of microcapsules added to each well containing an explant (at 1.5 mg $well^{-1}$) and incubation together at 15°C until sampled. Samples were prepared in triplicate for each treatment per time point. Samples were recovered for observation at 1.5, 3, 6, and 24 h, and gently washed with PBS to remove any microcapsules adhering to the surface. The samples were fixed in Davidson's (freshwater) fixative overnight before being dehydrated through an ethanol series, infiltrated with paraffin and subsequently embedded. The blocks were sectioned at 5µm using a microtome (Microm HM340, Germany), mounted on glass slides and stained with Mayer's haemotoxylin and eosin using an automated stainer (Shandon Linistain GLX, USA), all according to standard histological procedures. Prepared sections were examined under a compound microscope equipped with fluorescent illumination (Olympus BH2, Japan) to allow observation at the appropriate wavelength for FITC (495 nm) and transport of microcapsules through intestine was evaluated visually.

2.3.5.3. Systemic distribution of orally administered microcapsules *in vivo*

Lyophilised FITC-labelled microcapsules were combined with ground commercial feed crumble in a 1:100 ratio (w/w). The mixture was combined with distilled water (2:1 w/w) to form a paste, which was extruded and dried at room temperature, and re-crumbled to achieve appropriate sized feed particles. Atlantic salmon fry, weighing approximately 1.0 g each, were fed to satiation using the prepared feed. Five fish were collected at 1.5, 6, 12, 24, 48 and 72 h post-prandium and lethally anaesthetised. Intestine, liver, kidney and spleen were collected from each fish, fixed and prepared for microscopic examination, and observed as in section 2.5.2 to detect any fluorescence characteristic of administered FITC.

2.3.6. Statistical analysis

Results were expressed as mean ± S.E. IBM *SPSS* Statistics (v20) was used for all statistical analyses. Regression-based curve estimation was used to determine protein content of unknown samples. Non-linear regression was used to calculate protein concentrations. Analysis of Variance (ANOVA) was used to compare differences between treatments, using Levene's Test to verify homoscedasticity. Tukey's Post-Hoc test was used to determine significantly different treatments, with results considered statistically significant at $P \leq 0.05$

2.4. Results

2.4.1. SEM analysis:

Less than 1% of microcapsules examined (8 of 827) exhibited any loss of structural integrity, such as incomplete capsule formation or obvious surface degradation. Microcapsules presented a regular spherical shape, with irregular surface and no obvious surface porosity (Fig. 2.1).

Image analysis of scanning electron micrographs of single unbroken microcapsules (n=819) indicated that the majority of the alginate microcapsules produced measured between 250 and 750 nm in diameter (Fig. 2.2). All samples demonstrated a tendency for microcapsules to form superficial aggregates.

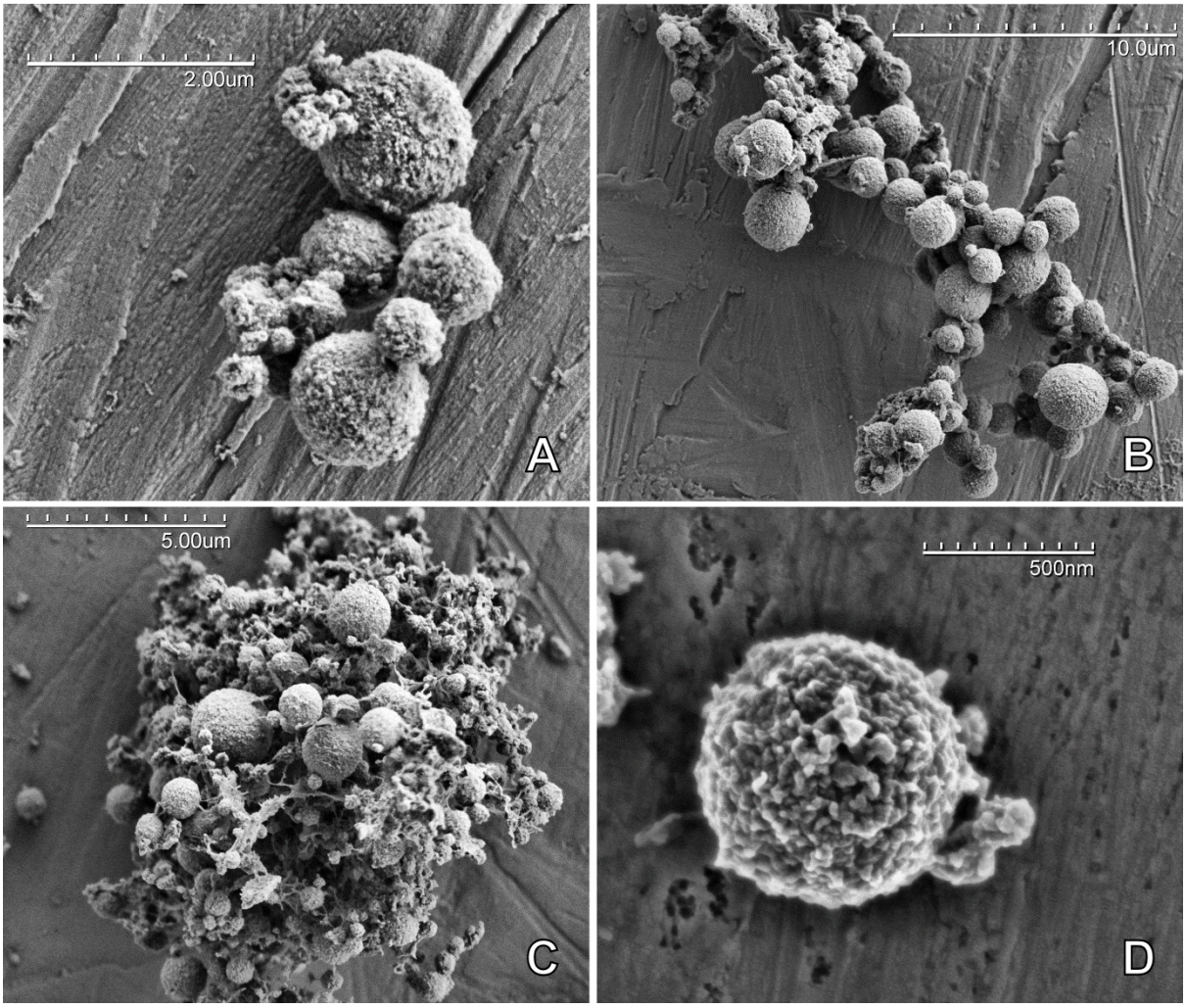

Figure 2.1: Scanning electron micrographs of BSA-loaded alginate microcapsules, showing examples of various aggregates (A, B and C), and detail of an individual microcapsule (D)

2.4.2. Protein Encapsulation:

Mean loading efficiency, determined by analysis of residual BSA after formation of microcapsules was 51.2%, as determined by comparison of a 50% dilution of supernatant to a quadratic curve fitted to blank-corrected absorbance values of BSA standards.

In calculating entrapment efficiency of microcapsules, the expected BSA quantity per sample was 30.7 µg. Mean quantity of BSA liberated on disintegration of microcapsules was 19.7 µg. Mean entrapment efficiency was 64.3% (±0.03).

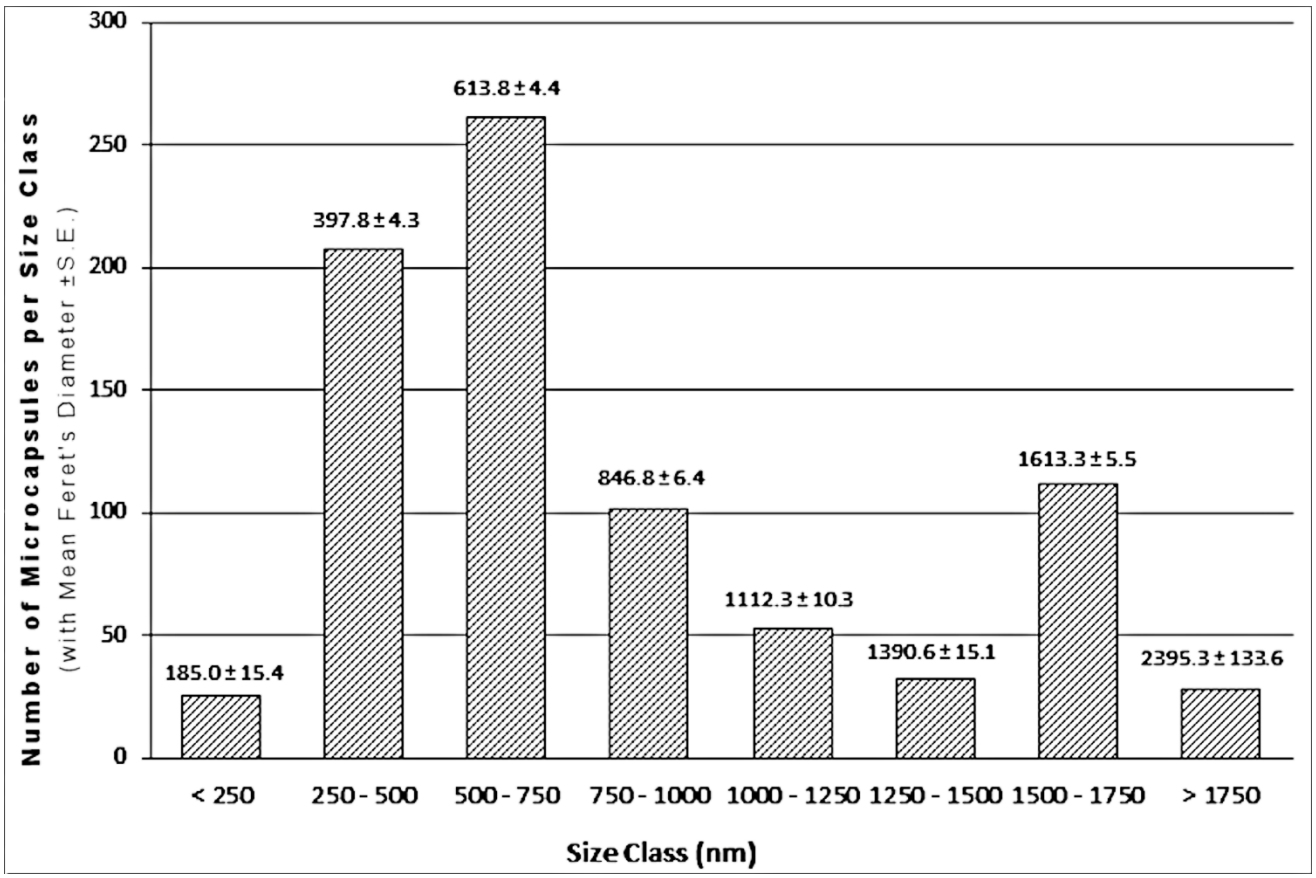

Figure 2.2: Frequency distribution of BSA-loaded alginate microcapsules in various size classes (nm) as determined by image-analysis of SEM (n=819), with Mean ±S.E. (nm) for each size class

2.4.3. Protein Release:

Release of protein from microcapsules in suspension was highest at pH 9, with quick initial release of 26.6 g mL-1 (17.7 μg mg-1) in 1 hour, which increased over time to 39.9 μg mL-1 (26.6 μg mg-1) after 24 h. Protein release was lowest at pH 4.5, with a maximum release after 24 h of 16.5 μg mL-1 (11.0 μg mg-1). At neutral pH, an initial release of 4.7 μg mL-1 (3.1 μg mg-1) in the first hour increased gradually, with a maximum of 21.9 μg mL^{-1} (14.6 μg mg^{-1}) of protein released after 24 h (Fig. 2.3). There was a significant difference between treatments at every time point (1h: F=744.81, $P < 0.001$; 3h: F=4465.33, $P < 0.001$; 6h: F=2801.95, $P < 0.001$; 24h: F=420.14, $P < 0.001$), with the release of BSA at pH 9 being consistently higher than the other treatments over time.

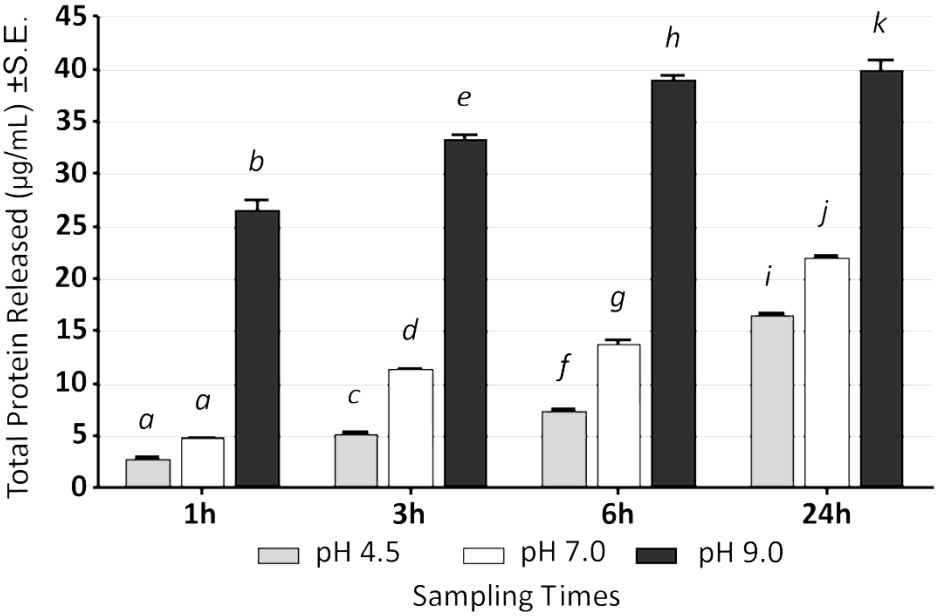

Figure 2.3: Cumulative protein release over time at 15 ˚C under the influence of different pH levels. Samples were collected at 1, 3, 6 and 24h. Lowercase letters indicate significant differences at each time point

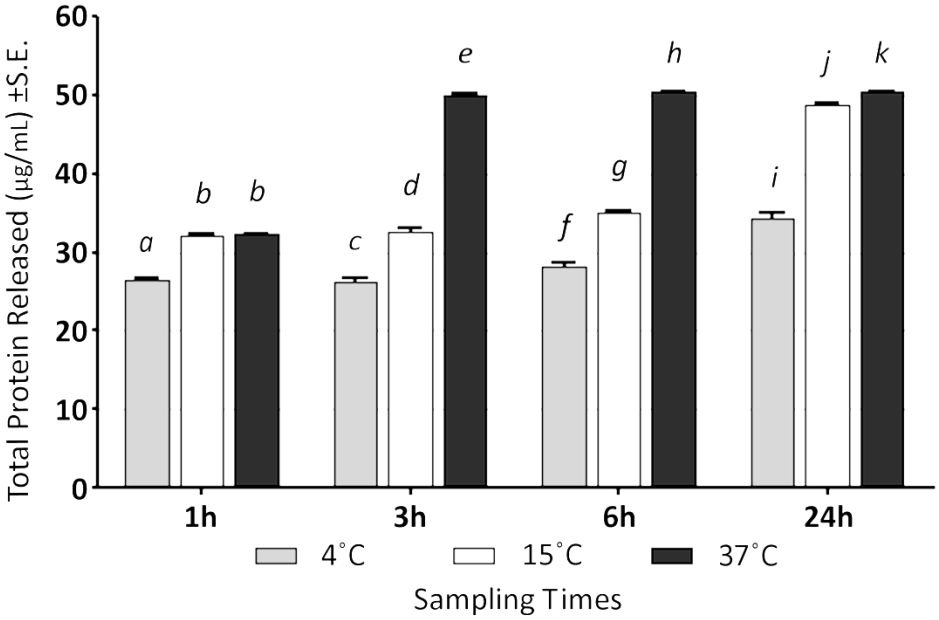

Figure 2.4: Cumulative protein release over time at pH 7 under continuous exposure to different temperatures. Samples were collected at 1, 3, 6 and 24h. Lowercase letters indicate significant differences at each

Initial release of protein at 4˚C (26.6 µg mL^{-1} or 17.7 µg mg^{-1}) was significantly lower than at 15˚C (32.2 µg mL^{-1} or 21.5 µg mg^{-1}) and 37˚C (32.4 µg mL^{-1} or 21.6 µg mg^{-1}) after 1 hour. After 3 hours, the amount of protein released at 37˚C increased to 50.0 µg mL^{-1} (33.32 µg mg^{-1}) and did not

increase significantly after that. Samples at 4˚C and 15˚C exhibited a gradual increase in protein released over time, reaching a maximum of 37.1 μg mL^{-1} (24.7 μg mg^{-1}) and 41.5 μg mL^{-1} (27.7 μg mg^{-1}) respectively after 24 hours (Fig. 2.4).

There were significant differences between temperature treatments across all time points (1h: F=1296.13, $P < 0.001$; 3h: F=923.03, $P < 0.001$; 6h: F=1679.71, $P < 0.001$; 24h: F=532.57, $P < 0.001$). All three treatments were significantly different from each other at all time points except 1 hour, where no significant difference was found between protein release at 15 and 37˚C.

1.1.1. Lysozyme bioactivity assay

The loading efficiency of the microencapsulation process was 43.17%. The expected measure of effective lysozyme activity units in eluted samples was 269.5 Units mL^{-1}. The actual level of mean lysozyme activity in samples, determined through turbidimetric observation, was equivalent to 268.1 ± 2.7 Units mL^{-1}, indicating no significant change in activity of lysozyme due to microencapsulation.

2.4.4. *Ex vivo* and *in vivo* uptake of fluorescent-labelled alginate microcapsules in *S. salar*

Uptake of fluorescent-labelled alginate microcapsules through epithelial tissue of excised intestine was clearly visible using optical microscope with fluorescent illumination (Fig. 2.5). When compared to SEM-based size determination, the samples demonstrating uptake indicated larger structures than expected. This may suggest uptake of microcapsule aggregates as opposed to individual microcapsules, which is concurrent with the tendency of microcapsules to aggregate as observed during SEM characterisation. Examination of images obtained at greater magnification (Fig. 2.4 C), indicated the presence of segmentation and apparent lacunae in the fluorescent particulate structures visible, further suggesting that microcapsules may have been taken up in aggregated clusters instead of individually.

Though control samples exhibited some auto-fluorescence, focused high-intensity fluorescence characteristic of fluorescent microcapsules was not found, in clear contrast to other samples. Control samples with direct addition of unencapsulated FITC in solution (Fig. 2.4 B) showed no differences in fluorescence compared to those control samples that had no FITC or microcapsules added to them (Fig. 2.4 A). This indicates that unencapsulated FITC solution was not taken up and retained in intestinal epithelial cells, in contrast to the uptake and retention of microcapsules. Alginate microcapsules were taken up by intestinal epithelium within 1.5 hours of exposure and incubation (Fig. 2.4 C). They were retained for as long as 24 hours after exposure, in spite of obvious tissue degradation over 24 hours (Fig. 2.4 F).

These observations provided clear and direct evidence demonstrating microcapsules were successfully taken up by intestinal explants *ex vivo*. Translocation of microcapsules taken up from the luminal region into intestinal villa and lamina propria was also observed, demonstrating potential for systemic uptake of microencapsulated material. This potential was substantiated *in vivo*, where organs from fry administered with fluorescently labelled microcapsules via feed demonstrated distinct fluorescence up to 72 h post-prandium (Fig. 2.5).

Samples obtained from all fish administered with treated feed provided similar results. FITC-labelled microcapsules were observed in the gut lumen and intestinal epithelium of the posterior intestine at 1.5 h following oral administration with feed, and in the lamina propria at 6 h (Fig. 2.5 E). In samples taken beyond 12 h (not shown), free fluorescence was observed in association with microcapsules, indicating release of FITC-labelled BSA into surrounding tissues over time. Fluorescence was observed in all other organs sampled at 72 h post-prandium, even though discrete microcapsules were not seen, indicating systemic distribution of the microcapsule contents. Fluorescence was observed in the parenchymatous tissue of the liver (Fig. 2.5 F), associated with the haematopoietic cells of the kidney as well as the epithelial cells of renal tubules (Fig. 2.5 G) and appeared to be associated primarily with the haematopoetic regions in the spleen (Fig. 2.5 H). In

contrast, no such fluorescence was observed in control samples from fish receiving untreated feed (Fig. 2.5 A-D).

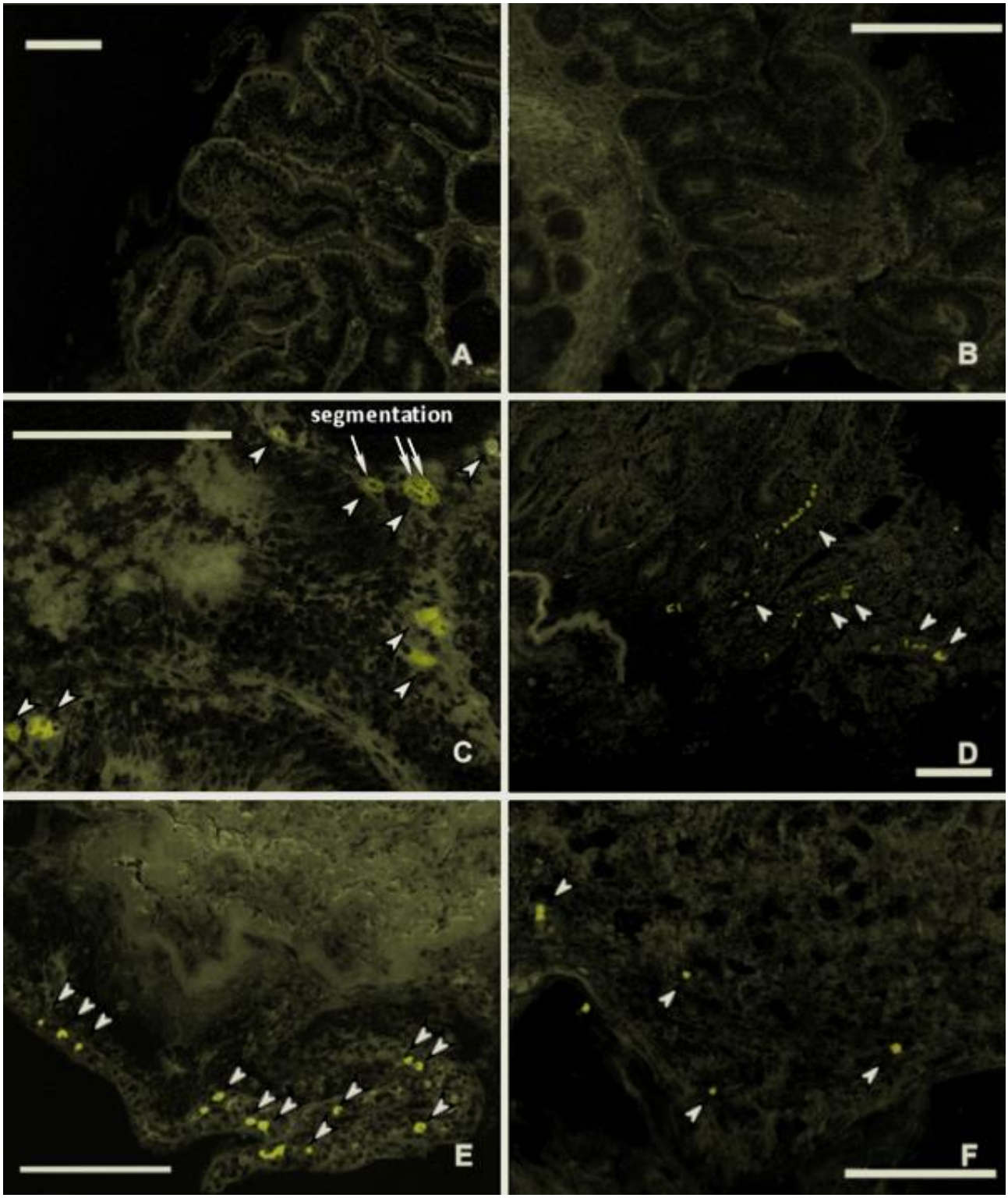

Figure 2.5: *Ex vivo* uptake of fluorescent-labelled alginate microcapsules in distal intestine of *S. salar*. Fluorescent optical micrographs of negative control samples (A and B), and of samples taken at 1.5 h (C), 3 h (D), 6 h (E) and 24 h (F) demonstrating microcapsule uptake (arrowheads). Segmentation indicating possible aggregated clusters indicated (C). Bar=100µm

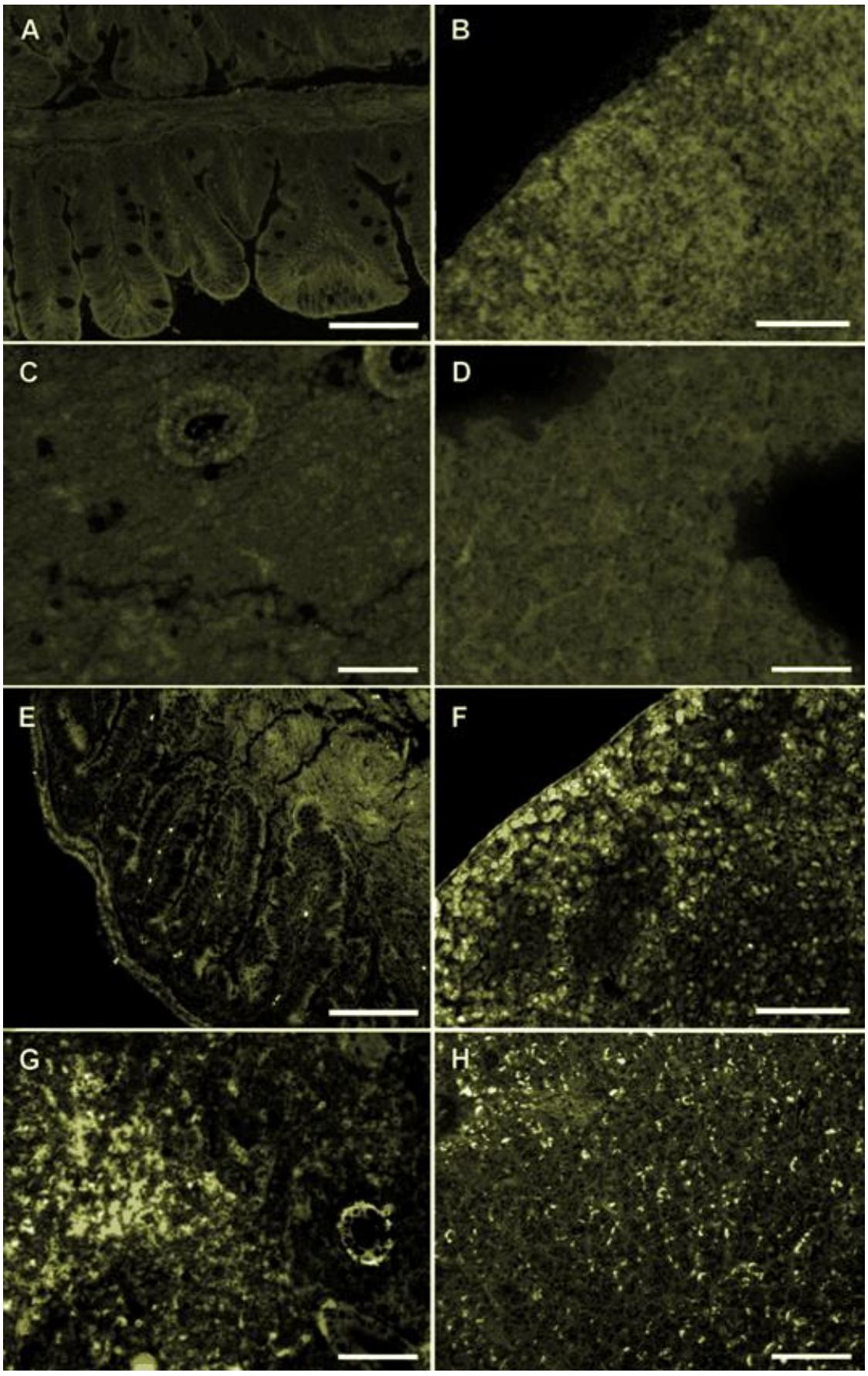

Figure 2.6: Fluorescent optical micrographs of *in vivo* samples from negative controls (A: Intestine, B: Spleen, C: Kidney, D: Liver), and from fish fed fluorescent microcapsules showing distinct fluorescence (E: Intestine, F: Spleen, G: Kidney, H: Liver). Bar=100µm

2.5. Discussion

This study examined the effectiveness of alginate microencapsulation for the delivery of immunoprophylactics to finfish through oral administration, assessing its ability to protect immunogens from digestive degradation and facilitate their uptake in the distal intestinal region. Loading efficiency, indicating the fraction of supplied protein incorporated into microcapsules, was determined using a subtractive approach comparing the amount of protein initially added to the amount remaining in suspension after microcapsule-manufacture was completed. In contrast, entrapment efficiency, reflecting the amount of protein liberated by disintegration of harvested microcapsules, indicated the actual protein content of microcapsules after any losses due to washing and collection procedures. The entrapment efficiency achieved by the process used was comparable to results from similar attempts to encapsulate model proteins in alginate [103, 104]. The difference between loading efficiency of the two proteins used may be related to the differences in molecular weight of BSA and lysozyme (66.5 kDa and 14.3 kDa, respectively), though other factors such as protein solubility and protein-polymer interactions may also have a considerable impact. Identifying the specific factors affecting such differences was beyond the focus and scope of the present study. However, this indicates an important area of investigation for further development of this approach, as properties of different materials could have an important impact on microencapsulation efficiency.

The impact of the process on immunogen integrity was also assessed by measuring changes in bioactivity of an encapsulated enzyme. The microcapsules produced demonstrated the ability to control the rate of release of encapsulated material under environmental conditions that were typical for the culture of temperate aquatic species. The microcapsules were of a size and shape that previous research has found to be well suited to oral administration and subsequent intestinal uptake in finfish [42]. The process was found to have no significant deleterious impact on bioactivity when used to encapsulate an enzyme. The microcapsules were found to be taken up readily by the distal

intestine, and *in vivo* administration demonstrated successful intestinal uptake and systemic distribution of contents.

The controlled release of encapsulated material in response to environmental conditions is an important factor that has led to the adoption of polymer microencapsulation in a variety of fields, including the delivery of immunoprophylactics [105-109]. Release dynamics of microcapsules loaded with bovine serum albumin (BSA) were assessed in this study at different pH levels based on the conditions typically found within the teleost gastrointestinal tract [110]. The distal intestinal region is the main target for microencapsulated immunoprophylactics orally administered to finfish, as it facilitates the uptake of particulate antigens and enables their presentation to the immune system [34, 36]. Release of BSA was lowest in an acidic environment, which corresponds to conditions characteristic of the teleost stomach, indicating the microcapsules were capable of preventing premature release of contents when administered to finfish orally. In contrast, release of BSA was greatest in mildly alkaline conditions that are typical of the distal intestinal region. The ability of these microcapsules to minimise release of contents through the early stages of the teleost GI tract, and release them readily in the target environment makes them ideally suited for oral immunoprophylaxis of finfish.

Release of BSA from the microcapsules was also examined at different temperatures, selected to address typical storage conditions (4˚C), culture conditions for the majority of temperate finfish species (15˚C), and to provide comparison with the mammalian applications from which the techniques used here were developed (37˚C). The microcapsules were well suited to storage at low temperatures, with the release of BSA being minimal at 4˚C. At temperatures characteristic of aquaculture conditions, the release of BSA was sustained and increased slowly over a 24 h period. This suggests the microcapsules are particularly well suited to immunoprophylactic applications, presenting the potential for prolonged exposure of the encapsulated antigen to the immune system and a consequently enhanced immune response as found in other research [111]. In comparison,

release of BSA from microcapsules was greatest at 37˚C, reaching its maximum within 3 h. This 'burst' release of the contents has impeded success in mammalian applications, leading to the preferential use of other polymers over alginate or the development of specific techniques to address this issue [112]. In the context of aquaculture applications, particularly for temperate species, this is not a problem as demonstrated by this study.

The impact of the microencapsulation process on encapsulated material is an important consideration when using microcapsules to deliver immunoprophylactic materials that may depend on intrinsic bioactivity to elicit immune responses. This has been investigated in other studies, where the excipients used in the microencapsulation process were found to have varying levels of negative impact on the integrity of encapsulated bioactive substances, particularly at the water-organic solvent interface [97, 113, 114]. The microencapsulation process used in this study was selected with a focus on minimising any potential deleterious effects on the encapsulated substance. This included low-impact mechanical methods, mild excipients and also contributed to the choice of alginate as the encapsulating material, which is known to be amenable to the preservation of bioactive substances [115]. Encapsulated lysozyme was used to determine the effect of the microencapsulation process, as its bioactivity is reliant on preservation of the integrity of its three-dimensional structure. In this context, the success of the microencapsulation strategy employed here is reflected by the absence of any measurable decline in the bioactivity of lysozyme post-encapsulation.

FITC-labelled microcapsules loaded with FITC-labelled BSA were orally administered to fish in this study to assess uptake of microencapsulated material by the epithelial tissue of the distal intestine, which is the final major hurdle in oral immunoprophylaxis prior to systemic distribution and presentation to the fish immune system. This study demonstrated the native ability of the teleost intestinal epithelium to take up particulates from the lumen and transfer them to the lamina propria *ex vivo*, independent of any supporting processes. The findings support earlier identification of fish

epithelial cells with characteristics of mammalian M-cells, which are responsible for particulate uptake and subsequent immune-presentation in mammals [40]. In contrast to fish, the intestinal uptake of particulates in mammals has been investigated in great detail, and direct evidence of the uptake mechanisms involved is readily available [116-119]. Following the administration of microcapsules with feed, the distribution of microcapsules and encapsulated contents to organs of immunological importance was clearly observed in this study, marked by distinct fluorescence in contrast to controls. Previously, the detection of corresponding gene expression in various organs of Japanese flounder, *Paralichthys olivaceus*, following enteric administration DNA-loaded alginate microcapsules provided indirect evidence of intestinal uptake in fish. Petrie and Ellis [42] provided evidence that anally intubated polystyrene microparticles up to 3µm in diameter were translocated to the kidney and spleen. Other researchers have also observed the phenomenon of antigen uptake in the teleost intestine and its subsequent systemic distribution [120, 121]. This study provides contiguous evidence of all the stages involved in successful delivery of an oral immunoprophylactic to fish, from oral administration in feed to uptake by intestinal epithelial cells and subsequent distribution to immunologically important organs. The similarity in the behaviour of the epithelial cells in the fish intestine and of mammalian M-cells as observed here implies that the strategies used to optimise oral delivery of immunoprophylactics to mammals may also be successful in fish. Future investigations in this area would benefit from the use of different labels to differentiate the microcapsule and its contents, thereby providing a clearer understanding of intestinal uptake and distribution dynamics in finfish and their implications on oral immunoprophylaxis strategies.

Size is an important determinant in the suitability of a microencapsulated preparation for oral immunoprophylaxis of fish, as are its shape, structural integrity and dispersibility. Smaller particle sizes increase uptake in the hindgut and subsequent systemic distribution [42], and decrease exposure to gastric degradation by accelerating gastric clearing rates [122-124]. The microcapsules produced in this study were all approximately spherical, maximising the possible volume of contents for a given microcapsule size. The microencapsulation process used here was modified to

produce microcapsules smaller than 5 µm, which has been found to be the upper threshold for successful uptake in mammalian studies [125]. The modal diametric distribution of between 250 and 750 nm achieved here was well below the target threshold size, which suggests excellent suitability for systemic distribution after successful intestinal uptake. The microcapsules produced were smaller than those manufactured using similar techniques [98], as well as those fabricated using more complex equipment, such as spray-drying [126, 127], membrane emulsification [128] and electrohydrodynamic atomisation [103]. The process used here achieved microcapsules that demonstrated no noticeable deformation under elevated electron bombardment, demonstrating structural robustness important for easy commercial applicability. The structural strength of the microcapsules is characteristic of the internal gelation process selected here, whereby sodium ions from the guluronic acid component are replaced with divalent calcium cations [129]. The resulting bonds are polar in nature, contributing to a charged surface that increases the aggregative tendency of the microcapsules observed in SEM examination [128]. A logical extension of the methods used in the present study for similar applications would be to neutralise surface charge, achievable by a variety of methods, including coating the microcapsules with a secondary polymer [130]. The resultant increase in dispersibility would have important implications for uniformity of immunoprophylactic dosage.

1.1.1. Conclusions:

This study established that an emulsion/internal gelation-based method, requiring no specialised equipment, can be employed successfully to fabricate protein-loaded alginate microcapsules suitable for oral delivery of immunoprophylactics to fish. The microcapsules produced were comparable to those obtained using other methods with respect to size, shape and controlled release of the encapsulated material. They demonstrated potential for delivery of a range of immunogenic material by virtue of having no measurable negative impact on bioactivity of an encapsulated substance. Direct visual evidence obtained here of the uptake of particulates by intestinal epithelium of fish, and of systemic translocation of these microcapsules when orally supplied, supports the

viability of oral immunoprophylaxis as an alternative to current disease management strategies used for fish.

2.6. Acknowledgements

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CHAPTER THREE

Microencapsulation of a putative probiotic *Enterobacter* species, C6-6, to protect rainbow trout, *Oncorhynchus mykiss* (Walbaum), against bacterial coldwater disease

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Keywords

Coldwater disease · *Flavobacterium psychrophilum* · Microencapsulation · Disease control · Oral delivery · Probiotic

3.1. Abstract

Flavobacterium psychrophilum is the causative agent of bacterial coldwater disease (BCWD), which has a major impact on salmonid aquaculture globally. An *Enterobacter* species, C6-6, isolated from the gut of rainbow trout, *Oncorhynchus mykiss* (Walbaum), has been identified as a potential probiotic species providing protection against BCWD. This study examined the effects of alginate microencapsulation on the protective efficacy of C6-6 against BCWD *in vivo* when administered to rainbow trout fry orally or by intraperitoneal (IP) injection. Viable C6-6 bacteria were microencapsulated successfully, and this process (microencapsulation) did not significantly deteriorate its protective properties as compared with the administration of non-microencapsulated C6-6 bacteria. Both oral and IP delivery of C6-6 achieved significantly better protection than control treatments that did not contain C6-6 bacteria. The highest relative percent survival (RPS) resulted from IP delivery (71.4%) and was significantly greater than the highest oral RPS (38.6%). Successful intestinal colonisation was not critical to protective effects of C6-6. The study showed that C6-6 administration, with or without encapsulation, was a viable choice for protecting fry from BCWD especially when administered intraperitoneally.

3.2. Introduction

Flavobacterium psychrophilum is a finfish pathogen that causes bacterial coldwater disease (BCWD) and rainbow trout fry syndrome (RTFS), which are responsible for high mortalities in salmonid aquaculture. Rainbow trout, *Oncorhynchus mykiss* (Walbaum), a major aquaculture species, are particularly affected by the pathogen, with major implications for the industry [131]. Bacterial coldwater disease may be successfully treated with antibiotics. However, negative impacts of antibiotic administration on stock and the environment, such as the emergence of antibiotic-resistant strains in fish farms make this a sub-optimal strategy for aquaculture disease management [132-135]. Development and implementation of immunoprophylaxis to prevent diseases such as BCWD has received great impetus due to the difficulty in medicating diseased fish and the risks associated with chemotherapeutic strategies [52, 53]. While some work has been done towards

development of effective vaccines against *F. psychrophilum* infection, there are currently none commercially available [136, 137].

The use of biological agents such as probiotic bacteria, to inhibit pathogenesis and subsequent detriment to stock, presents an alternative strategy for aquaculture disease management. While definitions have evolved over time, probiotic bacteria may be considered as being live micro-organisms that, when administered in adequate amounts to a host organism, confer a health benefit to the host [138]. This health benefit may manifest in various forms including disease control through production of inhibitory compounds, enhancement of the immune response of the fish, or through outcompeting pathogens for nutritional and spatial resources [46, 139]. Autochthonous bacteria among the microbial flora inhabiting fish skin, gills and intestine have been studied as a form of protection against fish pathogens with some success [140-143]. Isolating probiotic strains from the host has proved an efficient method of selecting effective probiotics [144-147].

The most commonly proposed mode of action for probiotic protection is the ability to colonise the intestine, thereby either forming a barrier against pathogenic bacteria or stimulating the host's immune system [148-150]. Adhesion of probiotic bacteria to the intestinal mucosa has been shown to enhance their antagonistic activity against pathogens [151-154]. It is therefore necessary to ensure successful delivery of an appropriate dose of the probiotic to the intestine of the host species. In the context of commercial scale aquaculture this would most conveniently be achieved via oral administration. A key consideration in this process is preservation of probiotic viability for optimal colonization of the intestine and limitation of any potential environmental or digestive degradation. Microencapsulation has been proposed as a useful method of achieving this, wherein probiotic cells are encapsulated at high density in a polymer like alginate to physically and chemically protect the microorganisms [155]. The use of microencapsulation strategies has been successfully demonstrated in aquatic species, such as the administration of a probiotic to Senegalese sole, *Solea senegalensis* Kaup [see 156]. Polymers have been extensively tested for use in such microencapsulation-based strategies; alginate is a widely used polymer microencapsulant due to its

resistance to proteolysis, high biocompatibility, ease of availability and relatively low cost, as well as its intrinsic potential as an immunostimulant [86, 87].

Several potential probiotic species have been identified in studies for their protective or inhibitory effects against pathogenic fish diseases. Examples include inhibition of yersiniosis in rainbow trout [147] and Atlantic salmon, *Salmo salar* L., [see 157], vibriosis in halibut, *Hippoglossus hippoglossus* (L.) [see 158] and in Atlantic salmon [157], and haemorrhagic septicaemia in common carp, *Cyprinus carpio* L. [see 159]. However, while many such probiotic candidates are initially identified by their *in vitro* inhibitory abilities against pathogens, this does not always translate to adequate *in vivo* protection [160, 161].

An *Enterobacter* species, C6-6, recently isolated from the intestine of rainbow trout has been shown to decrease mortality due to BCWD [162]. It is non-pathogenic to fish, and demonstrated inhibition of *F. psychrophilum* growth *in vitro* [142]. When administered orally *in vivo*, it improved survival of rainbow trout challenged with *F. psychrophilum*, and has consequently been proposed as a probiotic species to control BCWD. When injected intraperitoneally (IP) in subsequent studies, C6-6 provided better protection against BCWD than previously achieved using oral administration [163]. While the exact mechanism of protection afforded by C6-6 is not understood, these results indicate that its protective properties may not be entirely dependent on successful colonisation of the host intestine, as expected for most probiotic bacteria [47]. Further, if colonisation is important when supplied orally, environmental effects and digestive degradation in the stomach may have a considerable negative impact on the final viability of C6-6 available for colonisation of the intestine. It would follow that enabling greater colonisation of the gut by protecting orally administered C6-6 from digestive degradation may result in increased protection of fish from coldwater disease.

The aims of this study were to assess any differences in the protective effects of C6-6 against coldwater disease achieved by:

- microencapsulation in alginate to protect cells during administration and transit through the gastrointestinal tract;
- administration via either oral delivery or IP injection

3.3. Materials and Methods

3.3.1. Bacterial culture

All C6-6 cultures were grown at 24°C in tryptone soy broth or agar (TSB/TSA; BD Diagnostic Systems, USA). TSA plates were inoculated aseptically with previously isolated frozen stocks of C6-6 and incubated for 24 h. Plate culture was used to inoculate 200 mL of TSB, which was incubated for 18 h with gentle mechanical shaking. TSB culture was cooled to 4°C over 1 h and then centrifuged at 1500x*g* for 10 min at 4°C. Centrifuged C6-6 pellet was suspended as required in phosphate buffered saline (PBS) for preparation of treatments.

F. psychrophilum challenge utilised a known virulent strain [259-93; 164, 165], which was grown in tryptone yeast extract salts media (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% calcium chloride, 0.05% magnesium sulphate, pH 7.2). Frozen stocks of *F. psychrophilum* were used to inoculate TYES agar plates, which were incubated at 15°C for 96 h. Several isolated colonies from plate culture were used to inoculate 15 mL of TYES broth, which was incubated for 48 h at 15°C with gentle mechanical shaking. This culture was then used to inoculate TYES broth (1% v/v) and maintained at 15°C for 72 h before use in challenge protocols.

3.3.2. Alginate microencapsulation of C6-6

C6-6-loaded alginate microcapsules were prepared using a method adapted from Hansen *et al.* [166]. Briefly, 300 mg sodium alginate salt (medium viscosity, Sigma-Aldrich) was dissolved in 9 mL distilled water by stirring with a magnetic stirrer (Corning, USA) at high speed. To this solution, 1 mL suspension of C6-6 bacteria from culture (in PBS; $OD_{525}\approx2.35$) was added and

stirring was continued for 10 min to ensure cells were homogenously dispersed through alginate solution to form the aqueous phase. To form the oil phase, 0.25 mL Tween-80 (Sigma-Aldrich) was thoroughly dispersed in 50 mL canola oil. The aqueous phase was gradually introduced into the oil phase while under constant magnetic stirring at approximately 900 rpm, which was continued for 20 min to achieve a water-in-oil emulsion. A gelling agent was produced by emulsifying 0.3 g Tween-80 and 0.459 g calcium chloride (dissolved in 5 mL distilled water) in 35mL canola oil. A total volume of 16 mL of the gelling agent was added drop-wise to the alginate-oil emulsion while stirring continued, which facilitated hardening of alginate microcapsules through calcium-crosslinking.

3.3.3. Microcapsule morphology and bacterial viability assessment

The emulsion was broken by the addition of 20 mL 0.05 M aqueous calcium chloride solution and the mixture was centrifuged at 300 x*g* to collect microcapsules. Recovered microcapsules were then washed twice with distilled water and 250 µg of washed microcapsules were resuspended in 1 mL distilled water by vortexing in a 2 mL centrifuge tube for 1 min. This suspension was applied to the surface of 12.6 mm diameter aluminium SEM mounts (ProSciTech, Australia) that had been pre-cooled to -80^{0}C, at 100 µL per mount. The loading surfaces of several SEM mounts were scored with parallel lines using a micro-scalpel prior to cooling. Immediately following application of microcapsule suspension to one scored mount, another empty scored mount was placed upside down on the loaded surface such that scored lines on one mount were at 45^0 to the other, and the pair was clipped together. Loaded stubs were removed to -80^{0}C immediately following loading and maintained for 6 h. Paired mounts were then shear-separated by sliding adjacent surfaces along each other to break microcapsules trapped in scored lines. All microcapsule-loaded mounts were lyophilised over 12 h and then sputter coated with carbon before examination by scanning electron microscopy (SEM; Zeiss Supra 35 SEM, Germany) at the Electron Microscopy Center, University of Idaho. Microcapsules were randomly selected from scanning electron micrographs (n = 750) and examined for size and morphology. Images were manually inspected to ensure they represented

single, unbroken microcapsules. Feret's diameter of individual microcapsules was recorded using ImageJ, a public domain Java image processing program [99].

Samples for viability assessment were prepared in triplicate, with 100 mg of washed microcapsules per sample added to 15 mL vials containing 9.9 mL (1% aqueous) sodium citrate solution each. Vial contents were immediately mixed by vortexing vigorously for 1 min. The suspension was incubated at 4°C for 10 min, vortexed vigorously for 10 s and then enumerated on TSA plates as per Chen *et al.* [167]. Plates were incubated at 24°C for 18 h and discrete colonies counted to determine colony forming units (CFU) mL^{-1} of microcapsule suspension, and thereby calculate CFU mg^{-1} of microcapsules.

3.3.4. Fish source and maintenance

Apparently healthy rainbow trout (*O. mykiss*) fry, with a mean weight of 0.1 g and no known previous exposure to pathogens, were obtained from the University of Idaho's Aquaculture Research Institute (ARI), Moscow, ID, USA. They were acclimated in 500 L tanks supplied with de-chlorinated, single-pass, municipal water at 14°C for three weeks on commercially available trout feed (Rangen, Idaho, USA). After acclimation, fry at a mean weight of 0.5 g were randomly allocated to one of 30 identical tanks, with 29 fish per tank.

3.3.5. Preparation of oral treatments

Commercially available pellet feed for rainbow trout was used for all oral treatments. Unbroken microencapsulated C6-6 emulsion was added in a 1:10 ratio by weight to feed and stirred thoroughly to coat feed with C6-6-loaded microcapsule emulsion (C6-6-µ-Oral). Coated feed was stored at 4°C until use. Total microcapsule yield was found to be 5.3% of total emulsion weight, giving 5.3 mg of microcapsules per gram feed.

Control treatments, summarised in Table 3.1, were prepared by coating feed using 1 mL non-microencapsulated C6-6 suspended in PBS (OD_{525}≈2.35) dispersed in 76.25 mL canola oil (C6-6-non-µ-Oral), blank microcapsule (not containing C6-6) emulsion (blank-Oral), and a placebo

control consisting of feed coated in only canola oil (con-Oral). The same additive to feed ratio was used in all applicable preparations.

Bacterial content of C6-6-non-µ-Oral samples was determined by enumeration on TSA plates in triplicate to determine CFU mL^{-1} as described. C6-6-µ-Oral samples were subjected to citrate digestion of the alginate microcapsules by adding equal volumes of 0.1 M aqueous sodium citrate solution, vortexing thoroughly at high speed for 1 min, and being allowed to stand for 10 min at 4°C. Digested samples were then enumerated by plating as for C6-6-non-µ-Oral samples.

Table 3.1: Summary of immunoprophylactic oral and IP treatments (and controls) administered prior to challenge with *F. psychrophilum*, and abbreviated labels used in text

Treatment	**Delivery method**	**Group Label**
Microencapsulated C6-6*	Oral	C6-6-µ-Oral
Non-microencapsulated C6-6	Oral	C6-6-non-µ-Oral
Blank microcapsules	Oral	blank-Oral
Control/Placebo (Feed only)*	Oral	con-Oral
Micro-encapsulated C6-6*	IP injection	C6-6-µ-IP
Non-encapsulated C6-6	IP injection	C6-6-non-µ-IP
Blank microcapsules	IP injection	blank-IP
Control/Placebo (PBS only)*	IP injection	con-IP

* = Allocated an extra challenge control (mock-infected) tank

3.3.6. Preparation of Intraperitoneal (IP) injection treatments

Microcapsule emulsion was broken and microcapsules recovered as in section 2.3. Washed C6-6 microcapsules were suspended in PBS (OD_{525}≈0.20) by vortexing for 1 min (C6-6-µ-IP). Control treatments, summarised in Table 3.1, comprised of non-microencapsulated C6-6 suspended in PBS (C6-6-non-µ-IP), and blank microcapsules (blank-IP) suspended in PBS, to equivalent optical densities(OD_{525}≈0.21 and OD_{525}≈0.22 respectively), and a placebo control using only PBS for

injection (con-IP). Samples of each suspension containing C6-6 were enumerated on TSA plates in triplicate to determine CFU mL^{-1} as described. Prior to plating, the microcapsule suspension samples were subjected to citrate digestion as described (Section 2.5).

3.3.7. Treatment administration

Fry were maintained for 3 days at 14°C in 9 L tanks and fed commercially available pellet trout feed prior to commencement of treatment administration. Treatment groups consisted of microencapsulated C6-6 bacteria or appropriate controls (Table 3.1), and were administered either orally or via intraperitoneal (IP) injection. Each treatment group was comprised of three replicate tanks. An additional tank was allocated to microencapsulated oral (C6-6-µ-Oral), microencapsulated IP (C6-6-µ-IP), oral control (con-Oral) and IP control (con-IP) treatment groups, which remained unchallenged with pathogen (mock-infection/challenge controls).

Feed was withheld from fish for 24 h before treatment administration. Oral treatments were administered at an actual feed weight of 3% of total biomass per tank per day for a 7-day period. IP injected treatments were administered to coincide with Day 1 of oral treatment administration. Prior to injection, fish were placed in a 2 L bath containing a mild anaesthetic, 50 ppm tricaine methanesulfonate (MS-222; Argent, USA), until loss of swimming equilibrium occurred. Injected treatments were administered at 25 µL per individual intraperitoneally (IP), and fish were placed in a recovery bath before being returned to the appropriate tank. IP injected fish were fed commercially available pellet trout feed at 3% of total biomass per tank per day for a 7-day period matching the duration of oral treatment administration. At the end of the 7-day period feed was withheld for 24 h from all fish before bacterial challenge was administered by subcutaneous injection of pathogenic *F. psychrophilum*.

3.3.8. Sampling

Two randomly selected fish were removed from each tank prior to treatment administration and euthanised by an overdose of MS-222. Kidney, spleen and liver samples were inoculated onto TSA

and TYES agar plates. Intestines were removed aseptically and flushed with PBS to remove any residual organic debris, homogenised with 1 mL PBS and inoculated onto TSA plates to screen for presence of C6-6 bacteria. TSA plates were incubated at 24°C for 24 h and TYES plates were incubated at 15°C for 96 h. Two fish from each tank were similarly sampled immediately prior to commencement of bacterial challenge with pathogenic *F. psychrophilum*. Presence of C6-6 was determined by subculturing and characterising any colonies that grew on TSA plates. Any colonies with morphology corresponding to C6-6 were isolated, followed by gram-staining and characterising through oxidase and catalase reactions [168]. Throughout the post-challenge period, reisolation of *F. psychrophilum* was attempted from 20% of daily mortalities per tank by streaking TYES plates with kidney, spleen and liver samples. *F. psychrophilum* was identified by characteristic colony morphology on TYES agar.

3.3.9. *F. psychrophilum* bacterial challenge

Eight days after commencement of treatment administration, challenge controls (mock-infected) were established by anaesthetising fish from the four challenge control tanks as described, and injecting them subcutaneously with 25 µL of PBS posterior to the adipose fin. All other fish were challenged with 25µL of previously prepared *F. psychrophilum* (strain 259-93) culture at 1.2 x 10^7 CFU per fish, injected similarly. Feeding was resumed 72 h post-challenge with treated or untreated diets corresponding to pre-challenge feeds. Tanks were monitored for mortalities, which were sampled as described, for 28 days post-challenge. Cumulative percent mortality (CPM) from each treatment was used to calculate the relative percent mortality (RPS) as:

RPS = (1 – (mean treatment group CPM/control group CPM)) x 100

3.3.10. Statistical analysis

Results were expressed as mean ± S.E.M. IBM *SPSS* Statistics (v21) was used for all statistical analysis. Analysis of Variance (ANOVA) or Independent-Samples T-test was used to compare differences between treatments as appropriate, using Levene's Test to verify homoscedasticity.

Tukey's Post-Hoc test was used to determine significantly different treatments, with results considered statistically significant at $p \leq 0.05$. A Bonferroni correction was set to allow for multiple curve comparisons for survival curve analysis, which was performed using the Log-rank test.

3.4. Results

3.4.1. Microcapsule morphology and bacterial viability assessment

No obvious loss of structural integrity, such as incomplete capsule formation, breakage, or surface porosity, was visible. Mean diameter was 22.7 ± 0.1 µm. Microcapsules were spheroidal with irregular dimensions, and many displayed noticeable flattening. Microcapsules loaded with C6-6 bacteria showed discrete spheroidal inclusions approximately 1 µm in diameter. This was consistent with expected size of individual C6-6 cells. Several of these inclusions were clearly observed in microcapsules that had been fractured prior to lyophilisation (Fig. 3.1), indicating successful incorporation of C6-6 bacteria within the microcapsule structure.

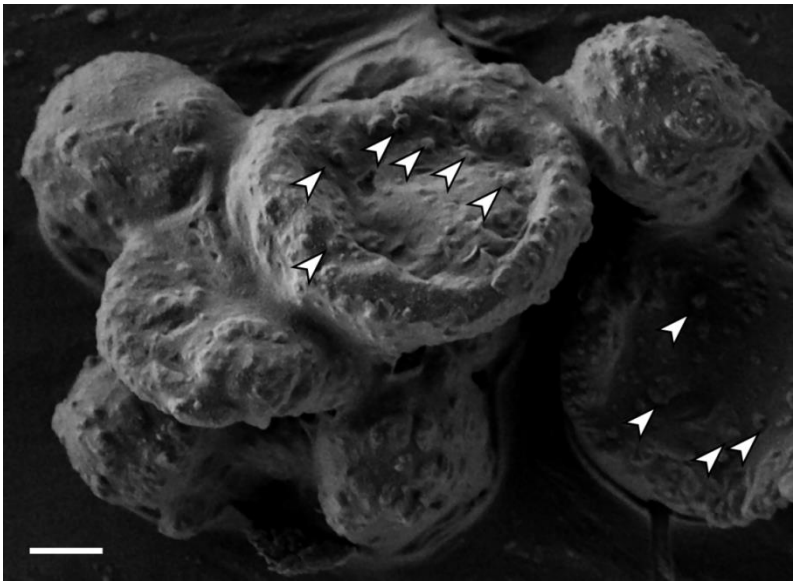

Figure 3.1: Scanning electron micrographs of C6-6 - loaded alginate microcapsules. Arrowheads indicate individual C6-6 bacterial inclusions in broken microcapsule (Bar = 5 µm)

In preliminary tests, 10 min of exposure to citrate buffer at 4°C resulted in approximately 10-fold reduction in viability of C6-6 (data not shown). Accounting for this decrease, mean bacterial viability of microencapsulated C6-6 was 2.8 x 10^6 CFU mL^{-1} of microcapsule suspension (in citrate

buffer), or 2.8 x 10^5 CFU mg^{-1} of microcapsules. Viability of C6-6 bacteria in all treatments is presented in Table 3.2.

Table 3.2: Viability of C6-6 bacteria in treatments and total approximate dose per fish over a seven day period prior to bacterial challenge with pathogenic *F. psychrophilum*

Treatment	**Viability**	**Mean total dose (bacteria/fish)**
C6-6-µ-Oral	1.5 x 10^5 bacteria g^{-1} of feed	0.6 x 10^6
C6-6-non-µ-Oral	1.3 x 10^6 bacteria g^{-1} of feed	4.2 x 10^6
C6-6-µ-IP	1.4 x 10^8 CFU mL^{-1}	0.7 x 10^7
C6-6-non-µ-IP	1.3 x 10^9 CFU mL^{-1}	6.5 x 10^7

3.4.2. Bacterial screening and reisolation

None of the fish sampled prior to administration of treatment showed any evidence of C6-6 or of previously existing *F. psychrophilum* infection.

Table 3.3: C6-6 bacteria reisolation from fish in different treatment groups receiving C6-6 via oral or IP routes*

Treatment	Reisolation success	Treatment	Reisolation success
C6-6-µ-Oral	66.7%	C6-6-µ-IP	0%
C6-6-non-µ-Oral	83.3%	C6-6-non-µ-IP	16.7%

* Six fish sampled per treatment, immediately prior to bacterial challenge with pathogenic *F. psychrophilum*

Prior to commencement of the bacterial challenge, C6-6 was successfully reisolated 24 h after cessation of feeding from the intestines of fish sampled from the C6-6-µ-Oral and C6-6-non-µ-Oral groups. One of the fish from the C6-6-non-µ-IP group also tested positive for C6-6 (Table 3.3). None of the samples produced any colony growth characteristic of *F. psychrophilum*, confirming the lack of any preexisting infection.

Post-challenge, *F. psychrophilum* was successfully reisolated from the kidney, liver or spleen of 87.1% (189/217) of all mortalities sampled.

3.4.3. *F. psychrophilum* challenge

No mortalities were observed in any of the mock-infected (challenge control) tanks. All other tanks registered mortalities by 28 days post-challenge.

CPM among oral treatments ranged from 57.3% (C6-6-non-µ-Oral) to 93.3% (blank-Oral) (Fig. 3.2). The C6-6-non-µ-Oral group showed the best challenge protection (RPS = 38.6%), and CPM was significantly lower than in the blank-Oral and con-Oral groups. Though CPM in the C6-6-µ-Oral group was not significantly greater than in the C6-6-non-µ-Oral group, it did not differ significantly from CPM in the blank-Oral and con-Oral treatments either. Though there were no significant differences in the CPM of fish in the blank-Oral and con-Oral groups, differences in survival curves between the treatments were significant (Fig. 3.3). Lowest level of protection achieved by controls in both oral and IP treatments was the same, but highest level of protection achieved through IP administration of C6-6 was significantly better than achieved by oral administration ($t = 2.85$, $df = 4$, $p < 0.05$).

Among the IP treated groups, CPM ranged between 26.7% (C6-6-non-µ-IP) and 93.3% (con-IP) (Fig. 3.2). CPM in the control (con-IP) group was significantly higher than the other three IP treatments. CPM in the blank-IP group was also significantly higher than in the C6-6-non-µ-IP group (RPS = 71.4%), which had the least mortalities. Microencapsulation did not produce significantly different CPM relative to non-microencapsulated IP treatment in the two treatments containing C6-6 (C6-6-µ-IP and C6-6-non-µ-IP).

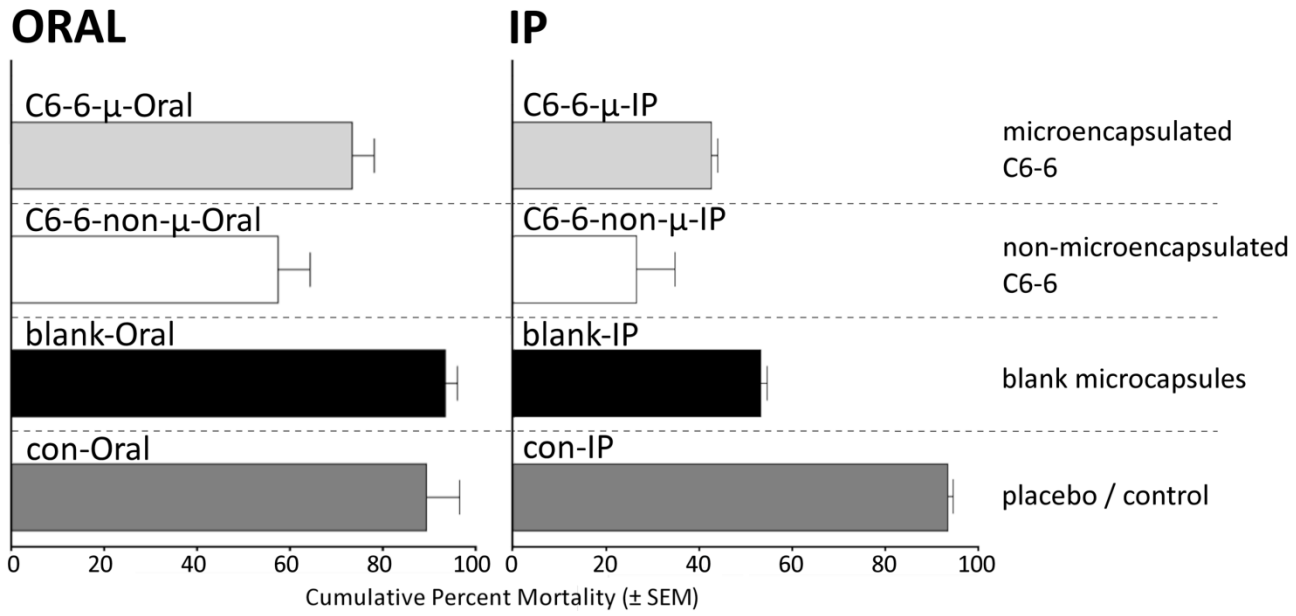

Figure 3.2: Cumulative Percent Mortality 28 days post-challenge with *F. psychrophilum* in rainbow trout

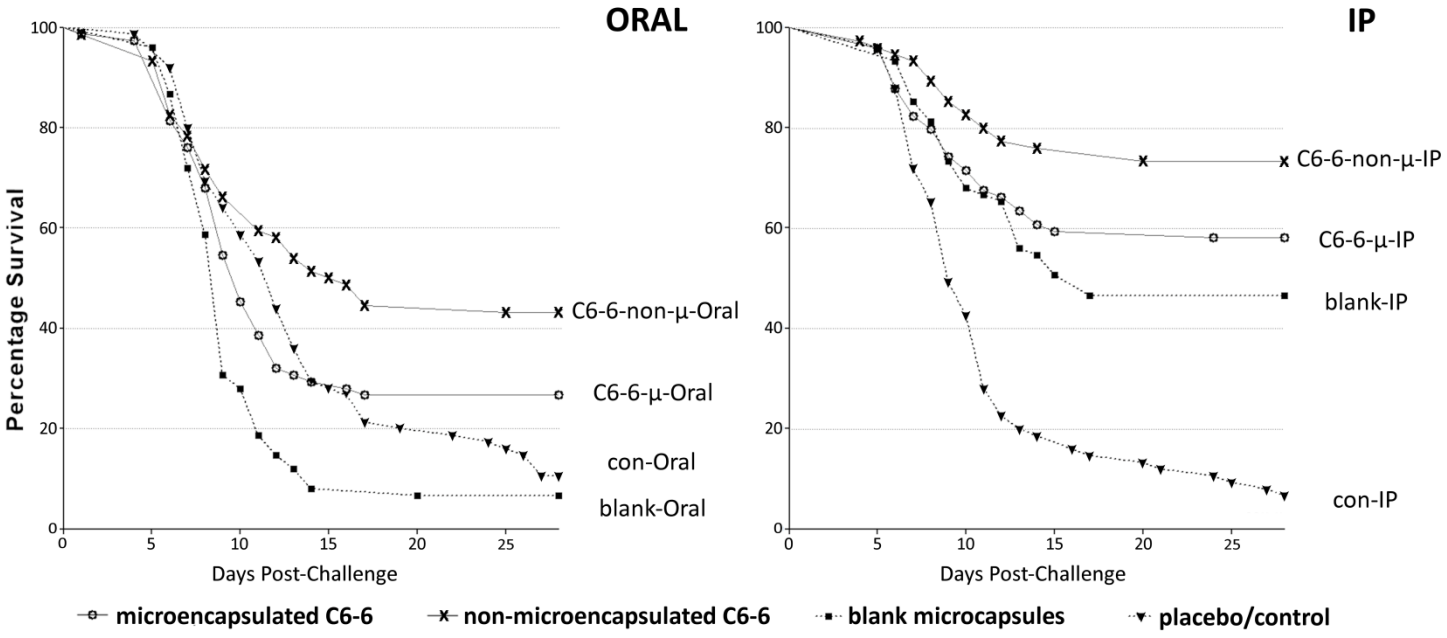

Figure 3.3: Cumulative Percent Survival 28 days post-challenge with *F. psychrophilum* in rainbow trout administered C6-6 treatments via oral and IP routes

CPM in the C6-6-µ-IP group did not differ significantly from the blank-IP group either. Analysis of survival curves showed a similar trend, with survival being significantly different for the control in comparison to all other treatments. In contrast to CPM data, survival curve analysis showed C6-6-µ-IP and C6-6-non-µ-IP groups to be significantly different from the blank-IP group (Fig. 3.3).

3.5. Discussion

This study examined the effects of alginate microencapsulation on C6-6, an *Enterobacter* species found autochthonously in the intestine of rainbow trout. This *Enterobacter* species has been shown to reduce mortality associated with coldwater disease [162]. The methods used here proved suitable for encapsulating live C6-6 bacteria and maintaining their viability through oral and IP administration protocols. The study assessed the comparative protection afforded to rainbow trout against BCWD through administration of C6-6 bacteria via oral delivery or IP injection. Any effect of the microencapsulation process on efficacy of each of those methods of administration was also assessed. The subcutaneous challenge protocol used here was selected over infection models that more closely simulate natural *F. psychrophilum* infections (such as immersion and cohabitation) as consistently achieving adequate mortality levels proved difficult in previous attempts with these models, which reflects the findings of other researchers [169]. Both oral and IP administration of C6-6 conferred some degree of protection against BCWD. However, alginate microencapsulation did not appear to confer any significant advantage in protective efficacy of C6-6 at the administered dose. In this study, orally administered C6-6 bacteria provided weaker protection than IP injected C6-6 treatments, the latter achieving significantly decreased mortality in fish challenged with *F. psychrophilum*.

Oral administration of C6-6 bacteria demonstrated significantly greater protection in comparison to control groups that did not receive C6-6 when fish were challenged with *F. psychrophilum*. C6-6 was successfully reisolated from intestines of the majority of fish that were supplied with C6-6 orally (C6-6-µ-Oral: 66.7%; C6-6-non-µ-Oral: 83.3%). In the absence of suitable methods for accurate quantification of C6-6 persistence in different treatments, no obvious differences between microencapsulated and non-microencapsulated treatment groups were observed. This implies that there may not be any significant advantage in shielding C6-6 from degradation with regard to improving protection from BCWD. However, the use of alginate microencapsulation in oral administrations may be advantageous in amplifying the immune response generated by C6-6. This

may explain the lack of a significant difference in CPM between the C6-6-µ-Oral and C6-6-non-µ-Oral treatments in spite of the difference in bacterial dose received by fish in the two groups. Although significantly greater than fish that did not receive C6-6, the highest level of protection achieved through oral administration was in the C6-6-non-µ-Oral group: RPS=38.6%. This was comparable to oral administration of C6-6 in an earlier study [170] where RPS was 45.8%. Together, these results indicate that oral administration of C6-6 was clearly beneficial but the relatively low RPS values indicate that feeding of C6-6 may not be suitable as the sole means of protecting stock against BCWD. However, this approach could be utilised as a supplementary or baseline treatment capable of reducing disease impacts in conjunction with other measures.

Intraperitoneally injected treatments achieved better RPS than comparable oral treatments. With the exception of a single fish, C6-6 could not be successfully reisolated from the intestine of any of the sampled IP-injected fish. This is not unexpected, given the lack of a direct connection between the intraperitoneal cavity and the GI tract, and the exceptional positive sample found may have been a result of error in the injection process. C6-6 administered via IP injection provided strong protection against BCWD, which was significantly greater than untreated controls. RPS achieved in the best protected IP group (C6-6-non-µ-IP) was significantly higher than the best protected orally treated group (C6-6-non-µ-Oral). This suggests that protection provided to rainbow trout by C6-6 against BCWD may not depend on successful colonisation of the intestine by C6-6 bacteria. Consequently, failure to significantly increase survival through the microencapsulated protection of orally delivered C6-6 compared to non-microencapsulated was not surprising, as any increase in dose viability and intestinal colonisation achieved did not automatically translate into better protection. The effectiveness of the IP administered C6-6 treatments in reducing mortalities indicates that C6-6 holds definite potential for development as an immunoprophylactic against BCWD in rainbow trout. This is further supported by recent findings that C6-6 was able to produce a cross-protective immune response after 28 days in rainbow trout when administered by IP injection, resulting in increased antibody titres against *F. psychrophilum* [163]. In contrast, protection conferred against *F.*

psychrophilum in the present study is unlikely to have been linked to increase in antibody titres due to the short interval between treatment administration and bacterial challenge. The results in this study may be better explained by a strong non-specific immune response produced by the administration of C6-6 bacteria, which indicates considerable potential for the use of C6-6 as an adjuvant in combination with other bacterial vaccines for fish. Future challenge-based studies of C6-6 employing a pathogen other than *F. psychrophilum* could provide valuable insight in this area. However, it is difficult to make specific recommendations for C6-6 as either an adjuvant or as an alternative vaccine against BCWD due to the gaps in our understanding of C6-6 protection mechanisms. Further characterisation of the immune response produced by C6-6 in rainbow trout over different treatment periods will assist and direct future optimisation of C6-6-based immunoprophylaxis against BCWD.

Though researchers have previously found dietary administration of alginate to fish resulted in an increased immune response [171, 172], oral administration of alginate alone (blank-Oral) did not increase survival here. This may be attributed to the comparatively low net amount of alginate made available to the fish, rather than the lack of immunostimulatory property. In contrast, the results from the IP treatments indicate that the alginate microcapsules may produce an immunostimulatory effect even when injected, as there was no statistical difference in CPM between the C6-6-µ-IP and C6-6-non-µ-IP groups even though the mean C6-6 dose per fish in the latter treatment was an order of magnitude greater than the former. The survival of fish in the blank-IP group was comparable to that of the C6-6-µ-IP group, further suggesting a systemic immunostimulatory effect of alginate that could be optimised through dose adjustment. Further research on the immune response elicited by alginate when supplied both orally and by injection to fish, and changes to this response over time, may contribute to a better understanding of the roles alginate could play in immunoprophylaxis of fish.

Morphological examination of microcapsules showed bacterial inclusions throughout the structure of the microcapsules, which indicated successful encapsulation of C6-6 cells within the alginate matrix as opposed to only surface association. The microcapsules were appropriately sized for administration with feed, and incorporated sufficient bacteria to produce a measurable protective response against BCWD in treated fish. In comparison to previous studies using C6-6 via oral administration [170], the oral dosage achieved in this study was somewhat lower. This could be addressed in the future by further concentrating the microcapsule content in the final emulsion used to coat feed. The typical focus of microencapsulating orally administered immunogenic substances is prevention of environmental and digestive degradation. Maintaining the viability of live cells adds an additional challenge. The encapsulant polymer, alginate, and the microencapsulation protocol used here were specifically selected and modified to minimise the impacts of the encapsulation process on C6-6 cell viability. In spite of this, CFU enumeration on agar media indicated approximately one log loss in viability. This may be attributed in part to the citrate-digestion method used to liberate encapsulated cells as found by Wadhawan *et al.* [173], though an attempt was made to approximate such losses. The encapsulation process may also have negatively affected amenability of cells to culture even though viability was preserved. This was not a possibility accounted for, as viability measurements were based on CFU establishment in this study. In this context, a more direct assay for assessing cell viability such as Wadhawan *et al.* [174], combined with studies focused on dose-response using C6-6, may provide more reliable estimates.

Though research has previously indicated the possibility of C6-6 secreting a substance that inhibits *F. psychrophilum*, the exact mechanism of protection has not been determined [142]. This makes it difficult to accurately identify the nature of the anti-BCWD protection conferred by C6-6. More work to identify the processes underlying the ability of C6-6 to protect fish from BCWD would be undoubtedly worthwhile. The results here support findings in previous studies that suggest C6-6 is a viable consideration for preventative management of *F. psychrophilum* infections and consequent BCWD outbreaks in rainbow trout. The evolving definitions of a probiotic make it difficult to

disqualify C6-6 from being considered a probiotic species in absolute terms. However, the results presented here concur with previous findings that indicate C6-6 provides stronger protection against BCWD in rainbow trout when administered by IP injection than when delivered orally. Consequently, it may be more appropriate to consider it a potential alternative vaccine and develop it further accordingly.

3.6. Acknowledgements

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CHAPTER FOUR

Assessment of immune response and protection against bacterial coldwater disease induced by a live-attenuated vaccine delivered orally or intraperitoneally to rainbow trout, *Oncorhynchus mykiss* (Walbaum)

This chapter formed a part of the manuscript:

Comparative protection achieved by mucosal immunisation of first-feeding Atlantic salmon, *Salmo salar* L., against yersiniosis by oral and immersion routes (Submitted for peer review; please see Appendix A)

B. Ghosh, T. D. Nguyen, P. B. B. Crosbie, B. F. Nowak, A. R. Bridle

4.1. Abstract

Flavobacterium psychrophilum is the causative agent of bacterial coldwater disease (BCWD), which has a major global impact on salmonid aquaculture. A candidate live-attenuated *F. psychrophilum* vaccine strain, CSF259-93B.17 (B17), has recently been developed. This study examined the protective efficacy of B17 grown in iron-limited conditions against BCWD in rainbow trout, when administered either orally or via intraperitoneal injection. B17 was administered via both routes with and without alginate microencapsulation, and comparative protective efficacy was assessed. The microencapsulation method used successfully encapsulated viable B17 bacteria. Fewer microencapsulated B17 cells were available per unit volume of vaccine compared to unencapsulated bacteria. However, protective efficacy under elevated challenge pressure was not significantly different for microencapsulated and unencapsulated B17 bacteria regardless of the route of delivery. Both oral and IP delivery achieved significantly better protection than controls. Orally administered B17 achieved protection levels comparable to those achieved by IP immunisation. Serum antibody production response was slower in orally immunised fish, but achieved similar titres to IP immunised fish prior to bacterial challenge. The study showed that B17 has potential as a vaccine candidate for the protection of rainbow trout from BCWD, and could present a viable disease control strategy even when administered orally.

4.2. Introduction

Bacterial cold water disease (BCWD) and rainbow trout fry syndrome (RTFS) are responsible for severe losses in global salmonid aquaculture and are caused by *Flavobacterium psychrophilum*, a Gram negative bacterium originally isolated from coho salmon, *Oncorhynchus kisutch* (Walbaum), in the U.S.A [175]. Rainbow trout and coho salmon are among the species most affected, and the rainbow trout farming industry is particularly impacted by *F. psychrophilum.* Clinical severity varies depending on strain virulence and fish size, with high mortality generally associated with smaller fish and fresh water stages [131].

Transmission of infection vertically from parent to progeny through eggs and ovarian fluid [176, 177] as well as horizontally in the water column has resulted in *F. psychrophilum* becoming a widespread pathogen in temperate and cold water finfish stocks. International trade in species susceptible to *F. psychrophilum* has extended its impact across the globe [131, 176]. Despite widespread aquaculture losses, no commercial vaccines are currently available and management of BCWD is primarily reliant on antimicrobial treatment [178]. The present ubiquity of *F. psychrophilum* and the risks associated with widespread antimicrobial administration make development of an effective preventative control method crucial.

Vaccination is important for the management of fish disease due to the difficulties in dispensing therapeutic measures to aquatic stock. Whole inactivated bacterins have been commonly used as fish vaccines against bacterial diseases. However, the process of bacterial inactivation employed may destroy or decrease efficacy of the antigens [54]. Inactivated or killed vaccines developed for protection against *F. psychrophilum* infection have thus far been limited and inconsistent, generally requiring an adjuvant to elicit an adequate immune response [179-183]. In humans and animal vaccines, these limitations have been successfully circumvented by the use of live pathogens that have been modified to attenuate virulence [184-186]. A number of studies have successfully used live-attenuated bacterial vaccines to elicit an immune response against infectious diseases in fish as well [180, 187-189]. A live-attenuated vaccine can potentially provide better protection than inactivated or subunit equivalents against later infection by the virulent pathogen without necessitating adjuvants, as the attenuated organism persists and metabolises within the host, and may even be capable of limited replication in the host in some cases [186, 190]. Immune responses elicited by such modified live vaccines are typically both cell-mediated and humoral and of greater magnitude and longer duration than those produced by inactivated or subunit vaccines [191].

A live-attenuated *F. psychrophilum* strain, CSF259-93B.17 (B17) has recently been developed [136]. Its virulence has been entirely attenuated following passage against increasing concentrations

of rifampicin. Intraperitoneal (IP) and immersion immunisation of rainbow trout with this live attenuated strain achieved significant protection when challenged with the virulent *F. psychrophilum* parent strain CSF259-93 [164, 165], though this was not the case with killed preparations of the bacteria [136]. In spite of this, relative percent survival (RPS) of fish immunised with B17 by immersion and by IP injection in initial trials was approximately 45%. Free iron available to a pathogenic bacterium within the host environment is typically lower than in commonly used growth media. Consequently iron-limited media (ILM) is thought to more closely resemble the pathogens' *in vivo* environment than common growth media, and thereby help conserve or boost pathogenicity. Several *F. psychrophilum* (CSF259-93) proteins found at higher levels *in vivo* than in typical (non-ILM) cultures were upregulated when the bacterium was cultured in ILM [192]. A recent study demonstrated enhanced efficacy when B17 was grown in iron-limited media (ILM) in comparison to non-ILM cultures [188].

Studies have shown that it is possible to achieve a significant level of protection from BCWD by IP administration of bacterin-based vaccines, particularly with an adjuvant such as mineral oil [178, 193]. However, there are cost-related and procedural drawbacks associated with injection vaccination under production conditions, as well as potential biological drawbacks like adverse impact on growth rate post-immunisation [71]. These make IP administration a sub-optimal vaccine delivery method for many commercial aquaculture operations. Fish are also required to be a minimum size before they can be injection vaccinated, which makes the method inapplicable for protection against the substantial mortalities associated with BCWD outbreaks in small fish.

Oral administration presents an ideal method for delivery of an efficacious vaccine to fish of any size, without the drawbacks associated with injection immunisation or handling required for most immersion delivery methods. However, oral vaccine trials have typically produced inconsistent results [72]. This is generally attributed to degradation of immunogenic material as it passes through the fish gastrointestinal (GI) tract before presentation to the immunologically active distal

portion of the hindgut [72, 90, 194]. The use of polymeric microencapsulation has been extensively studied in mammals as a means of protecting orally delivered immunogens [195]. A number of studies have had some success in achieving significant immune response through the use of microencapsulation in oral immunisation of fish as well [120, 196-200].

The aims of this study were to evaluate the relative protective efficacy of a live-attenuated *F. psychrophilum* strain, CSF259-93B.17 (B17), against BCWD when:

- administered orally, in comparison to intraperitoneal injection (IP) administration,
- microencapsulated in alginate prior to administration via oral or IP routes

4.3. Materials and Methods

4.3.1. Bacterial culture

All *F. psychrophilum* cultures were grown in tryptone yeast extract salts media (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% calcium chloride, 0.05% magnesium sulfate, pH 7.2), either as broth or as agar (1.5% w/w), using aseptic techniques. For immunisation treatments, a TYES agar plate was inoculated with frozen stock of *F. psychrophilum* CSF 259-93B.17 (B17), a rifampicin-attenuated non-pathogenic strain. Inoculated plates were incubated at 15°C for 72 h. Plate culture was used to inoculate 5 mL of TYES broth, which was incubated for 48 h at 15°C with mechanical shaking (approximately 85 rpm). Iron-limited media (ILM) was prepared by adding an iron-chelating agent, bipyridine (Sigma-Aldrich, St. Louis MO, USA), to TYES broth at a final concentration of 25 µM. ILM was inoculated with 1% (v/v) 48 h B17 culture and incubated for 72 h at 15°C with gentle mechanical shaking for use in immunisation treatments.

F. psychrophilum challenge utilised a known virulent strain [259-93; 164, 165]. Frozen stocks of *F. psychrophilum* were used to inoculate TYES agar plates, which were incubated at 15°C for 120 h. Several isolated colonies from plate culture were used to inoculate 15 mL of TYES broth, which was incubated for 48 h at 15°C with gentle mechanical shaking. This culture was used to inoculate 247.5 mL TYES broth (1% v/v) and maintained at 15°C for 72 h before use in challenge protocols.

Bacteria were harvested by centrifuging cultures for 10 min at 1500 x*g*. The supernatant was decanted and pellets were re-suspended in sterile phosphate buffered saline (PBS, pH 7.2). Bacterial concentration was initially approximated using optical density at 525 nm (OD_{525}), and subsequently confirmed by enumeration in triplicate on TYES agar plates using a previously established drop plate protocol [167] to determine CFUs mL^{-1}.

4.3.2. Microencapsulation of bacteria and viability assessment

4.3.2.1. Alginate microencapsulation of rifampicin-attenuated *F. psychrophilum* (CSF259.93B.17)

Alginate microcapsules containing rifampicin-attenuated *F. psychrophilum* (B17) were manufactured using a previously published method with modifications [201]. Briefly, 300 mg sodium alginate salt (medium viscosity, Sigma-Aldrich) was dissolved in 9 mL distilled water by stirring with a magnetic stirrer (Corning, USA) at high speed for 30 mins, and then cooled to 4˚C. Magnetic stirring of alginate solution was resumed and 1 mL bacterial suspension in PBS ($OD_{525} \approx$ 2.1), previously cooled to 4˚C, was added. Stirring was continued for 10 min at approximately 900rpm, facilitating homogenous dispersion of bacteria through alginate solution, to form the aqueous phase. The oil phase was produced by dispersion of 0.25 mL Tween-80 (Sigma-Aldrich) in 50 mL canola oil. The aqueous phase was gradually introduced into the oil phase under constant stirring, which was continued for 20 min at approximately 900 rpm to achieve a water-in-oil emulsion. Alginate microcapsules were hardened through calcium-crosslinking by the drop-wise addition of 16 mL of gelling agent (85% w/w canola oil, 14.15% w/w 0.7 M aqueous calcium chloride solution, 0.85% w/w Tween-80).

4.3.2.2. Bacterial viability assessment

For bacterial viability assessments, the final suspension was broken with the addition of 20 mL 0.05 M aqueous calcium chloride solution. Microcapsules were collected by centrifuging at 300 x*g* for 10 min at 4°C, and washed in PBS. Samples were prepared in triplicate, with 100 mg of washed microcapsules per sample added to 15 mL vials containing 9.9 mL (1% aqueous) sodium citrate

solution each. Vial contents were immediately mixed by vortexing vigorously for 1 min. The suspension was incubated at 4°C for 10 min, vortexed vigorously for 10 s and then enumerated in serial dilution on TSA plates as per previously established protocols [167]. Plates were incubated at 15°C for 96 h and discrete colonies counted to determine colony forming units (CFU) mL^{-1} of microcapsule suspension, and thereby calculate CFU mg^{-1} of microcapsules.

4.3.3. Fish maintenance, immunisation and sampling

4.3.3.1. Fish source, maintenance conditions, and experimental setup

Apparently healthy rainbow trout fry, with a mean weight of 0.1 g and no known previous exposure to pathogens, were obtained from the University of Idaho's Aquaculture Research Institute (ARI), Moscow, ID, USA. They were held in 500 L tanks supplied with de-chlorinated, single-pass, filtered municipal water at 14°C for three weeks, and fed a commercially available trout pellet feed (Rangen, Idaho, USA). Fish were grown to a mean weight of 3.1 g and then separated into treatment groups. Treatments consisted of intraperitoneal (IP) injection or oral administration of alginate-microencapsulated live B17, or an appropriate control treatment (Table 4.1). Each treatment group was comprised of 136 randomly selected fish held in a 500 L tank, with one tank allocated per treatment. On Day 56 of the trial, fish from each treatment were randomly allocated to one of 48 identical 9 L tanks, with 25 fish per tank. Each treatment group comprised of four replicate tanks, of which one was excluded from bacterial challenge (mock-infected) to provide challenge controls, as summarised in Table 4.1.

4.3.3.2. Oral immunisation

Oral immunisation was initiated on day 1 of the trial, coinciding with administration of intraperitoneal injection immunisation. Commercially available trout pellet feed was used to prepare all oral treatments. Unbroken microcapsule emulsion was mixed with feed at a ratio of 1:5 (w/w) and stirred thoroughly to coat feed with emulsion (B17-µ-Oral). Coated feed was prepared daily, and stored at 4°C until use. Using the same additive to feed ratio (1:5 w/w) control treatments were prepared by coating feed with non-microencapsulated B17 (B17-non-µ-Oral) or blank

microcapsules suspended in canola oil – PBS emulsion (blank-Oral), as summarised in Table 4.1. A placebo/treatment-control consisted of feed similarly coated with only canola oil-PBS emulsion (con-Oral). Feed was withheld from fish for 24 h prior to initiation of oral immunisation. Each oral treatment was administered at an actual feed weight of 3% of total biomass per tank per day over 54 days. To minimise potential oral tolerance, oral treatments were administered as per a staggered 18-day feeding regimen comprised of 7 days of treated feed followed by 11 days of untreated feed, repeated thrice before bacterial challenge.

Samples of microencapsulated bacterial emulsion were enumerated on TYES agar plates in triplicate to determine CFU mL^{-1}, but subjected to citrate digestion of microcapsules prior to plating. This was achieved by adding each sample to an equal volume of 0.1 M aqueous sodium citrate solution, which was vortexed at high speed for 1 min and allowed to stand for 10 min at 4°C before serial-dilution and plating. In preliminary tests, 10 min of exposure to citrate buffer at 4°C resulted in approximately 10-fold reduction in viability of B17 (data not shown), and this reduction was accounted for in calculating viability of bacteria post-microencapsulation. Samples of unencapsulated bacterial suspension used to prepare immunisation treatments were similarly enumerated on TYES plates, without inclusion of the citrate-digestion step.

4.3.3.3. Injection immunisation

Fish were injected intraperitoneally (IP) with microencapsulated B17 or one of three controls. Initial administration of treatments occurred on day 1 of the trial, and corresponded with the commencement of oral treatments. An identical booster immunisation was administered to coincide with commencement of the final 18-day oral immunisation regimen (day 37). To prepare microencapsulated IP treatment (B17-µ-IP), microcapsule emulsion was broken and microcapsules recovered as in section 2.2.2. Washed B17-loaded microcapsules were suspended in PBS ($OD_{525} \approx$ 0.39) by vortexing gently for 1 min. Control treatments were prepared by suspending non-microencapsulated bacteria ($OD_{525} \approx 0.42$; B17-non-µ-IP) or blank microcapsules ($OD_{525} \approx 0.31$;

blank-IP) in PBS, and are summarised in Table 4.1. A placebo/treatment-control was also prepared, using only PBS for injection (con-IP). Samples of each treatment containing bacteria were enumerated on TYES plates in triplicate to determine CFU mL^{-1}. Prior to plating, the microencapsulated treatment samples were subjected to citrate digestion as described in section 2.3.2.

Table 4.1: Summary of oral and intraperitoneally (IP) injected immunisation treatments (and controls) administered to rainbow trout before challenge with *F. psychrophilum*, and abbreviated labels used in text

Treatment	**Delivery method**	**Group Label**	**Fish/Tank**	**No. of Tanks**
Microencapsulated B17	Oral	B17-µ-Oral	25	3 (+1 mock infected)
Non-microencapsulated B17	Oral	B17-non-µ-Oral	25	3 (+1 mock infected)
Blank microcapsules	Oral	blank-Oral	25	3 (+1 mock infected)
Control/Placebo (feed coated with canola oil/PBS)	Oral	con-Oral	25	3 (+1 mock infected)
Micro-encapsulated B17	IP injection	B17-µ-IP	25	3 (+1 mock infected)
Non-encapsulated B17	IP injection	B17-non-µ-IP	25	3 (+1 mock infected)
Blank microcapsules	IP injection	blank-IP	25	3 (+1 mock infected)
Control/Placebo (PBS only)	IP injection	con-IP	25	3 (+1 mock infected)

Feed was withheld from fish for 24 h before immunisation. Prior to injection, fish were anaesthetised with 50 ppm tricaine methanesulfonate (*MS-222, Argent, Redmond, WA, USA) until loss of swimming equilibrium was evident.* Each treatment (25 µL; B17-µ-IP: 1.8×10^7 CFU $fish^{-1}$ and B17-non-µ-IP: 1.6×10^8 CFU $fish^{-1}$) was administered intra-peritoneally and fish were placed in a recovery bath before being returned to the appropriate tank. Commencing 24 h post immunisation, fish were fed untreated commercial pellet feed at 3% of total biomass per tank per day until challenged.

4.3.4. Sampling

Samples were collected before initial immunisation on day 1 and at day 19, 37 and 55. At each time point 12 fish were sampled from each treatment. Each fish was euthanised by lethally anaesthetising with an overdose of MS-222, and then exsanguinated by severing the caudal peduncle. Blood was collected in a 1.5 mL centrifuge tube and allowed to clot overnight at 4°C. The next day, samples were centrifuged (15000 x*g*) for 5 min and sera collected. Serum samples were stored at -80°C until used to evaluate immune response through an enzyme-linked immunosorbent assay (ELISA). Serum collected from fish prior to immunisation was pooled and used as the negative control for ELISA. Kidney, spleen and liver samples were also obtained from each fish, inoculated onto TYES agar plates and incubated for 96 h at 15˚C to test for any pre-existing *F. psychrophilum* infection.

Reisolation of *F. psychrophilum* was attempted from at least 20% of daily mortalities per tank throughout the challenge period by inoculating TYES plates with kidney, spleen and liver samples, and incubating for 96 h at 15˚C.

4.3.5. *F. psychrophilum* challenge

Bacterial challenge was initiated on day 57, at a mean fish weight of 9.7 g, following previously published methods [201]. Fish from the challenge control tanks were mildly anaesthetised as described, and injected with 50 µL of PBS subcutaneously posterior to the adipose fin. All other fish were similarly anaesthetised and challenged with 50 µL of previously prepared *F. psychrophilum* culture (at 8×10^8 CFU per fish) injected subcutaneously posterior to the adipose fin. Feeding with untreated commercial pellet feed was resumed 48 h post-challenge at 1% total biomass per tank per. Tanks were monitored for mortalities, which were sampled as described, for 21 days post-challenge. Cumulative percent mortality (CPM) from each treatment was used to calculate the relative percent mortality (RPS) as:

RPS = (1 – (mean treatment group CPM/control group CPM)) x 100

4.3.6. Enzyme-linked immunosorbent assay (ELISA)

An ELISA was used to determine antibody titers against *F. psychrophilum* in all groups of fish as per previously published methods [182], with an additional modification. A blocking step was added prior to serum application to reduce non-specific binding, wherein 150 µL of 5% (w/v) non-fat dry milk (Bio-Rad, Carlsbad, CA, USA) + potassium phosphate buffered saline (KPBS) + 0.05% (v/v) Tween-20 (Fisher Scientific, Pittsburgh, PA, USA) was added to each well and plates incubated at room temperature for 1 h. Serum samples were diluted from 1:200 to 1:12800 in a series of doubling dilutions in PBS containing 0.02% sodium azide. Titre was set as the reciprocal of the highest dilution with an optical density at least two times greater than the negative control.

4.3.7. Statistical analysis

Data were analysed at 11-days post-challenge (100% mortality in positive controls) and at 21-days post-challenge (trial termination). All statistical analysis was performed in IBM SPSS Statistics v21 [202]. Analysis of Variance (ANOVA) was used to compare differences in mean cumulative mortality between treatments. Differences in mean antibody titres were also analysed using ANOVA. Homoscedasticity was verified using Levene's Test. Tukey's Post-Hoc test was used to determine significantly different treatments, with results considered statistically significant at $P \leq 0.05$. Where data could not be transformed to meet ANOVA's assumptions, the level of significance was lowered to $P \leq 0.01$ to avoid falsely rejecting the null hypothesis [203]. Students T-test was used to determine any significant differences between mortality at 11-days and 21-days post-challenge for each treatment group, with results considered statistically significant at $P \leq 0.05$. Survival curves were analysed by the Kaplan-Meier method using the Cox-Mantel model, and multiple curve comparisons were performed with an adjusted significance threshold.

4.4. Results

4.4.1. Bacterial viability

B17 was successfully reisolated from citrate digested microcapsules, with isolates on TYES agar displaying characteristic yellow pigmented colonies, which indicated incorporation of bacteria into

microcapsules. Accounting for the 10-fold reduction in viability due to the citrate-digestion step, mean bacterial viability of B17 post-microencapsulation was 1.9 x 10^4 CFU mg^{-1} of microcapsules. Viability of B17 in microencapsulated oral immunisation treatment (B17-µ-Oral), calculated by citrate-digestion of unbroken emulsion, was 2.35 x 10^6 CFU g^{-1} of emulsion. Viability of B17 in PBS suspension ($OD_{525} \approx 0.39$) of microcapsules used to prepare IP immunisation treatment (B17-µ-IP) was 1.8 x 10^8 CFU mL^{-1}. Viability of bacteria in the different immunisation treatments and resultant doses are summarised in Table 4.2.

Table 4.2: Viability of B17 in immunisation treatments (±SD), and approximate dose administered over immunisation period

Group	Viability	Mean Dose (bacteria $fish^{-1}$)	No. of Doses
B17-µ-Oral	4.7 x 10^5 bacteria g^{-1} of feed	7.7 x 10^5	3
B17-non-µ-Oral	6.1 x 10^6 bacteria g^{-1} of feed	1.05 x 10^7	3
B17-µ-IP	1.8 (±0.17) x 10^8 CFU mL^{-1}	1.8 x 10^7	2
B17-non-µ-IP	1.6 (±0.30) x 10^9 CFU mL^{-1}	1.6 x 10^8	2

4.4.2. Bacterial screening and reisolation

None of the fish sampled prior to immunisation showed any evidence of *F. psychrophilum* infection. Yellow-pigmented bacterial colonies characteristic of *F. psychrophilum* were re-isolated from the kidney, liver or spleen of 92% (248/270) of all mortalities sampled post-challenge. In addition, mortalities exhibited clinical symptoms of BCWD including yellow-pigmented biofilm on the anterior body and necrotic lesions [204].

4.4.3. *F. psychrophilum* challenge

There were a total of six mortalities observed in the mock infected groups. *F. psychrophilum* could not be reisolated from any of these fish, and none showed any physiological or behavioural signs characteristic of BCWD.

Table 4.3: Cumulative Percent Mortality (CPM) and Relative Percent Survival (RPS) 11 days and 21 days post-challenge with *F. psychrophilum* in rainbow trout immunised with rifampicin-attenuated vaccine (B17) via oral and IP routes. Superscripts indicate significantly different CPM values between Oral or IP treatments at a particular time point. Preceding asterisk (*) indicates significantly different CPM values between time points for the same treatment group.

Treatment	**Day 11**		**Day 21**	
	CPM (± SE)	RPS	CPM (± SE)	RPS
B17-µ-Oral (Oral Microencapsulated B17)	86.67 [a] ± 3.53	13.33	92.00 [e] ± 2.31	8.00
B17-non-µ-Oral (Oral Non-Microencapsulated B17)	82.67 [a] ± 2.31	17.33	86. 67 [e] ± 1.33	13.33
blank-Oral (Oral Blank Microcapsules)	88.00 [a] ± 3.53	12.00	94.67 [e] ± 1.33	5.33
con-Oral (Oral Placebo)	100.00 [b] ± 0.00	-	100.00 [f] ± 0.00	-
B17-µ-IP (IP Microencapsulated B17)	*86.67 [c] ± 2.67	13.33	*92.00 [g] ± 0.00	8.00
B17-non-µ-IP (IP Non-Microencapsulated B17)	80.00 [c] ± 2.67	20.00	85.33 [g] ± 1.33	14.67
blank-IP (IP Blank Microcapsules)	94.67 [d] ± 2.31	5.33	97.33 [h] ± 1.33	2.67
con-IP (IP Placebo)	100.00 [d] ± 0.00	-	100.00 [h] ± 0.00	-

The challenge dose, initially estimated by optical density, was higher than anticipated and consequently resulted in greater mortality in all challenged groups than would be expected at a dose more closely resembling natural infection levels. Since mortality reached 100 percent in the placebo/treatment-control groups for both oral and IP administration (con-Oral and con-IP) 11 days post-challenge, this time point was selected for comparison of treatment effects. Among oral treatments, mean CPM ranged from 82. 7% (B17-non-µ-Oral; RPS: 17.3) to 88.0% (blank-Oral; RPS: 12), and from 80.0% (non-µ-IP; RPS: 20.0) to 94.7% (blank-IP; RPS: 5.3) among IP treatments (Table 4.3). CPM was significantly higher in the placebo/treatment-control group compared to all other treatments, but there were no significant differences in CPM observed between these other treatment groups. Analysis of survival curves over the 21 day challenge indicated a similar trend among oral treatments, with the con-Oral (placebo) group being significantly different from all other treatments, but with no significant differences between treatments (Fig 4.1). Analysis of survival curves among IP treatment groups showed no difference

between B17-µ-IP and B17-non-µ-IP groups, though both were significantly different from blank-IP and con-IP groups. Survival curves for blank-IP and con-IP (treatment-control) group were not significantly different (Fig 4.1).

As expected, treatment groups not immunised with bacteria (blank-Oral, con-Oral, blank-IP, con-IP) did not show any antibody titres. Oral treatments did not produce any measurable antibody titres at day 19 (Fig 4.2). Intraperitoneally-delivered B17 treatments (B17-µ-IP and B17-non-µ-IP) showed significantly increased titres at day 19 compared to control (pre-immunisation) serum levels, but did not differ significantly from each other. At day 37, all groups immunised with B17 showed significantly higher antibody titres compared to controls, but were not significantly different from each other. At day 55, antibody titre in the B17-non-µ-Oral group was significantly higher than in the B17-µ-Oral group ($t = 5.754$, $df = 4$, $P < 0.01$). Antibody titre at Day 55 in the B17-non-µ-IP group was similarly significantly higher than in the B17-µ-IP group ($t = 15.496$, $df = 4$, $P < 0.01$).

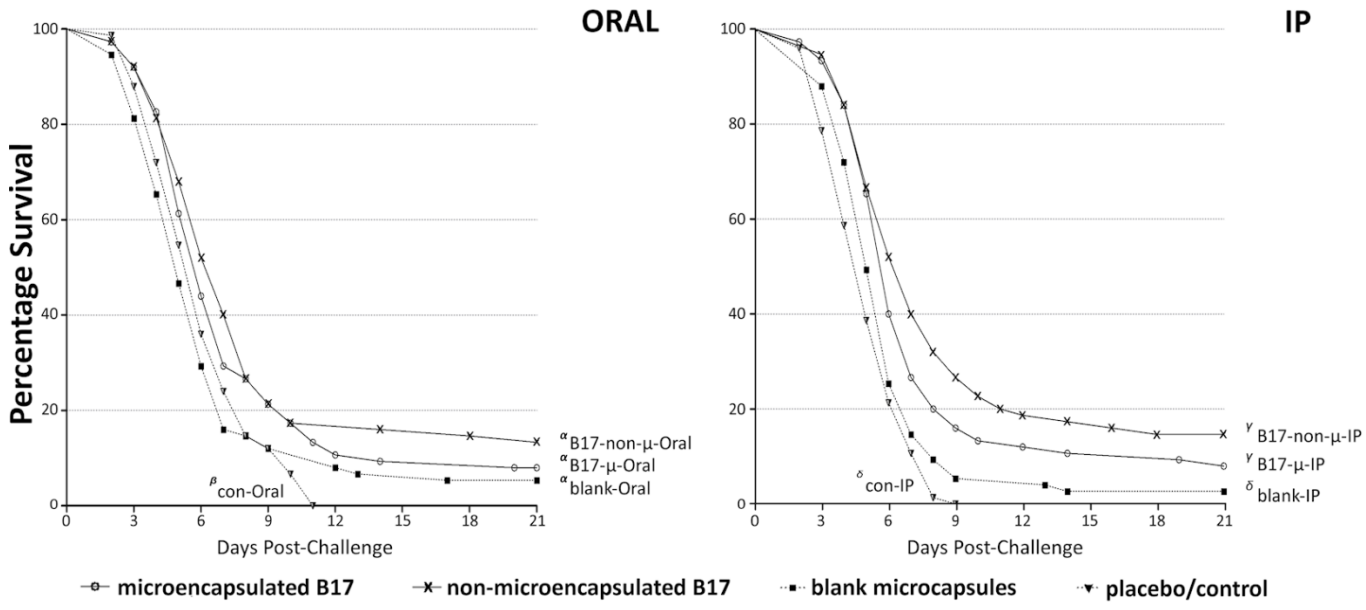

Figure 4.1: Survival of rainbow trout immunised with live-attenuated vaccine (B17) via oral and IP routes after subcutaneous challenge with *F. psychrophilum*. Preceding superscripts indicate significantly different treatments

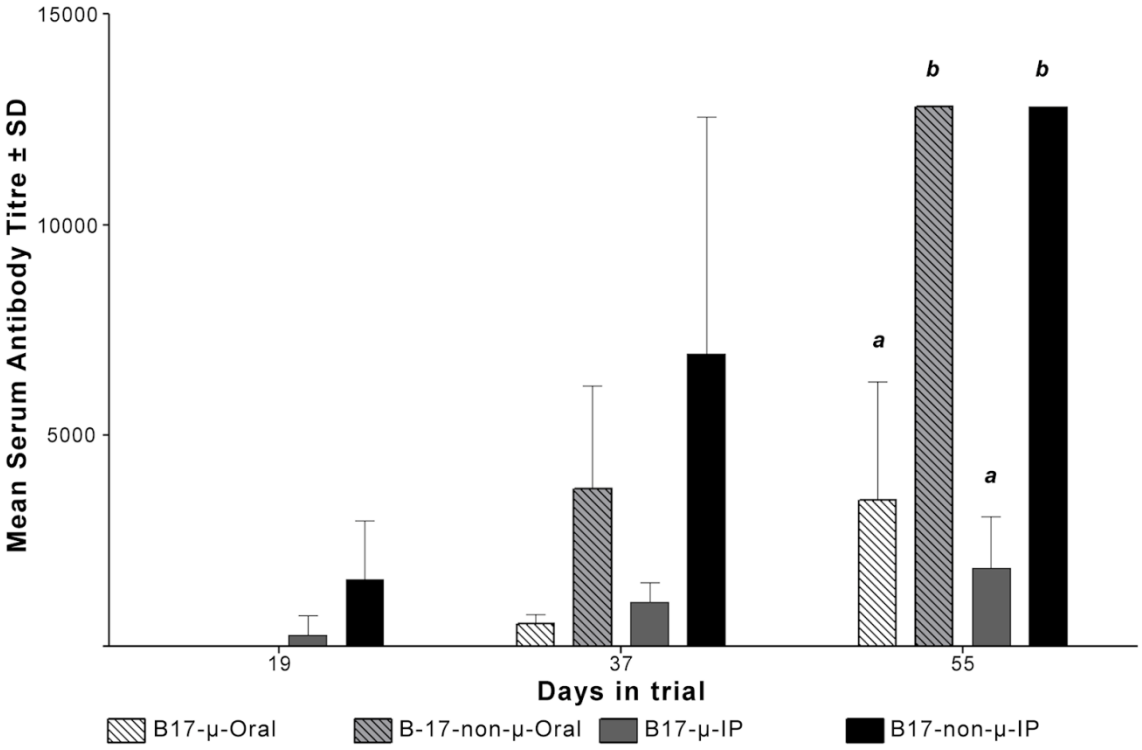

Figure 4.2: Mean serum antibody titres detected in samples collected at different time-points through the trial from B17-µ-Oral (orally administered microencapsulated B17), B17-non-µ-Oral (orally administered non-microencapsulated B17), B17-µ-IP (IP administered microencapsulated B17) and B17-non-µ-IP (IP administered non-microencapsulated B17) groups. Lowercase letters indicate significantly different treatments within a particular time point.

No significant difference was observed between the unencapsulated groups, B17-non-µ-Oral and B17-non-µ-IP, or between the microencapsulated groups, B17-µ-Oral and B17-µ-IP (Fig 4.2). Titres for both the groups administered unencapsulated B17 were at the maximum observable value across all replicates at day 55.

There were survivors in all treatment groups at the conclusion of the trial (21 days post-challenge) in spite of the elevated challenge pressure (Table 4.3). In all but one of the treatment groups, no significant differences were observed between CPM at 11 days post-challenge and at 21 days post-challenge. For the group injected with microencapsulated B17 (B17-µ-IP), the CPM at 21 days post-challenge was significantly greater than CPM at 11 days post-challenge ($t = 1.512$, $df = 4$, $P = 0.050$).

4.5. Discussion

This study assessed the comparative protection against BCWD achieved by oral and IP injection of B17, a live rifampicin-attenuated non-pathogenic strain of *F. psychrophilum* (CSF259-93B.17), grown under iron limited conditions. Previous studies have reported a decrease in immune response, or oral tolerance, linked to the extended administration of orally delivered antigens in various fish species including salmonids [205-207]. Accordingly, in this study the oral immunisation treatments were administered to fish as per a staggered regimen to minimise any such effects. The high challenge dose resulted in complete mortality in control groups by 11 days post-challenge and progressed at a greater rate in all untreated controls in this study than is typically observed in similar BCWD trials [188, 208]. In spite of these effects of elevated challenge pressure, this study demonstrated a significant level of protection against BCWD provided by the live-attenuated B17 vaccine in rainbow trout compared to non-immunised controls, and supports findings of previous studies [136, 188]. The study also demonstrated that under the conditions tested, protection provided by the candidate vaccine did not significantly differ between routes of administration.

Oral vaccine candidates tested for fish have typically produced inconsistent results [72, 209-212]. In contrast, in this study there was no significant difference in protection achieved through oral or IP administration of the vaccine. However, there were differences in the time course of the antibody response produced by the two routes of immunisation. At 19 days post-initial immunisation, orally administered treatments did not produce any measurable ELISA titres, while IP treatments did. Samples collected at 37 and 55 days after initial immunisation showed no significant difference in antibody titres for corresponding oral and IP treatments, indicating the relative delay in antibody response to oral B17 administration had been effectively bridged. This is not unprecedented, as assessment of immune responses to a bacterial vaccine administered via different routes to eels, *Anguilla anguilla* [213], and to rainbow trout [214], have demonstrated similar differences in kinetics of antibody response.

The lack of consistency in oral vaccine results is typically attributed to digestive degradation of the immunogenic material and consequent loss of antigenicity before reaching the distal portion of the hindgut, which has been identified as an immunologically active region involved in particulate antigen uptake [34, 72]. The use of polymeric microencapsulation has been explored at length as protection for antigens delivered to humans, terrestrial animals and to a limited extent, in finfish [74, 90, 215]. In this study, a low impact method was validated for microencapsulation of live B17 cells with alginate using an emulsion/internal gelation protocol. Similar methods have been used with success in encapsulating live cells and delivering them as antigens [156, 201, 216, 217]. The method used here achieved successful encapsulation of live cells without negating viability, as evidenced by the establishment of viable cultures after dissolution of alginate *in vitro*. The number of viable microencapsulated cells per unit volume of suspension was found to be lower than unencapsulated cells through enumeration of CFUs on agar media. This may be related to volumetric considerations of the effective particles in suspension, as the individual microcapsules were an order of magnitude larger than *F. psychrophilum* cells. As a consequence, fewer microencapsulated cells would be expected per unit volume of suspension than unencapsulated cells. The possibility of achieving higher doses could be investigated through concentration of microcapsules in the coating emulsion, or the identification of microcapsule saturation limits towards increasing the bacterial concentration in the microcapsules themselves. During culture of bacteria in ILM, observations of optical density did not necessarily correspond to CFUs of suspension in agar media based on non-ILM cultures. A similar phenomenon has been observed in *F. psychrophilum* cultured in the absence of nutrients [218, 219], indicating the possibility that not all viable cells cultured in restrictive media can be successfully cultured in agar media. When administered as a vaccine, though they produced significantly lower antibody titres than unencapsulated treatments immediately prior to challenge, microencapsulated treatments did not result in significantly lower protection. This may indicate protection arising from microencapsulation was less dependent on antibody response. A number of studies in mammals and

fish have observed non-specific immunostimulatory properties of alginate, suggesting adjuvant properties in addition to functioning as a protectant [87, 171, 220-222]. This could explain differences in antibody titre between microencapsulated and unencapsulated treatments not being reflected in mean CPM of corresponding groups. The ELISA results also indicate that B17 grown in ILM is capable of stimulating a strong systemic antibody response through both IP and oral administration, which is not typically the case with most oral vaccines for finfish [72]. The observations from this study suggest that, given enough time, orally administered B17 may be able to produce a systemic response comparable to that achieved through IP immunisation even when administered at a relatively lower dose. Further study involving dose response trails would undoubtedly provide valuable insight into the feasibility of B17 as an effective oral vaccine against BCWD.

Although mortality reached 100 percent in all treatment-control groups (con-IP and con-Oral) 11 days post-challenge, there were survivors in all groups that were immunised or received control treatments at the conclusion of the trial (21 days post-challenge). Mortality rates for all these groups decreased over the latter half of the trial. With the exception of one group (B17-µ-IP), there were no significant increases in mortality through this period in any of the treated groups. This indicates a possible continuation in the protective effect of the administered treatments, which was sufficient to prevent complete mortality in any of the treated groups in spite of the elevated challenge pressure. Since titre levels of all replicates in both B17-µ-IP and B17-µ-Oral groups were at the maximum observed value at day 55, extending the assay to examine maximum achievable antibody titres in the future may provide important information regarding the potential efficacy of ILM-grown B17 as a vaccine against BCWD in rainbow trout. Assessing the duration of protection achieved through B17 immunisation against *F. psychrophilum*, by incorporating a second challenge for survivors in a future study, would also provide valuable information regarding the practical potential for the use of B17 to manage *F. psychrophilum* infections in rainbow trout. The vaccine used in this study was grown in iron limited media (ILM) to maximise antigenicity based on previous findings of

increased pathogenicity in the same strain of *F. psychrophilum* when grown in ILM. A comparison of protective efficacy of ILM-grown B17, and of B17 grown in standard media, would also help elucidate the role of iron-limited growth conditions in its efficacy as a vaccine.

A subcutaneous challenge protocol was selected for use in this study over infection models that more closely simulate natural *F. psychrophilum* infections (such as immersion and cohabitation) as adequate and consistent mortality levels proved difficult to achieve in previous attempts with these models, which reflects the findings of other researchers [169]. The challenge dose administered in this study was higher than anticipated based on the optical density of the pathogenic culture, resulting in precipitous mortalities in all groups and 100% mortality of positive controls within 11 days of challenge with pathogen. The consequent loss in analytical resolution made it difficult to determine detailed differences in protection against BCWD between treatment groups, such as the relative change in survival of fish in the B17-µ-IP group through the latter half of the experiment. It also made it difficult to draw conclusions from the present study regarding potential protection levels achievable with a more natural challenge dose. However, the significant protection observed in groups immunised with B17 and the marked decrease in mortality rate over the trial period despite the high challenge pressure, combined with results from previous studies, provides further support for the efficacy of this B17 vaccine.

The threat of BCWD is a substantial one, potentially impacting not just salmonid industries globally, but also other economically important species like the ayu, *Plecoglossus altivelis* [223]. The ability to protect fish stocks effectively from the disease is critical to the future stability of these industries and the live-attenuated *F. psychrophilum* strain CSF259-93B.17 tested in this study demonstrates considerable potential as a viable solution. Considering the major impact of *F. psychrophilum* on small fish, where IP administration is inapplicable, reliance on an injected vaccine is insufficient as an industry-wide disease management strategy. An efficacious oral vaccine would be an important tool in any disease management strategy involving the culture of

small fish. This vaccine candidate's ability to provide oral protection that was comparable to IP injected treatments is a major factor that should be considered in assessing its commercial development prospects.

4.6. Acknowledgements

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CHAPTER FIVE

A quantitative real-time polymerase chain reaction assay for direct detection and absolute quantification of *Yersinia ruckeri* at low levels in fish spleen and faeces

5.1. Introduction

Yersinia ruckeri, a Gram-negative member of the family *Enterobacteriaceae*, is a major pathogen impacting aquaculture. First identified as the cause of yersiniosis or Enteric Redmouth disease (ERM) from rainbow trout (*Oncorhynchus mykiss* Walbaum) in the Hagerman Valley, USA [224], *Y. ruckeri* has been shown to cause disease in several species of farmed and wild fish [225-228]. It is now considered a ubiquitous pathogen that has been isolated from diseased fish, as well as other taxa and the environment, in locations around the world [229]. Salmonids are known to be particularly susceptible to *Y. ruckeri*, which is capable of causing mass mortalities, and the pathogen significantly impacts global salmonid culture. Clinical infections can be identified by haemorrhaging in and around the oral cavity, vent at and the base of fins, as well as blood spots in the eye and exophthalmia [230, 231]. *Y. ruckeri* has also been shown to establish subclinical infection in the distal intestine, resulting in asymptomatic carriers. These carriers act as pathogen 'reservoirs', transmitting infection horizontally through the water column via faeces and periodic intestinal sloughing, particularly when stressed [232]. Research has indicated that infection may be detectable in intestine and faeces prior to onset of acute systemic infection [233].

While vaccination has managed to protect fish to varying degrees against the effects of acute yersiniosis and subsequent mortality, it has been unable to prevent the establishment of asymptomatic carriers within surviving populations. Clinical expression from pre-existing subclinical infection has been reported in different asymptomatic salmonid populations up to two months after a clinical outbreak [232-235]. A method for accurate detection and precise quantification of subclinical infections in stock would therefore benefit disease management strategies considerably.

Pathogen detection has traditionally been achieved using culture-based methods in combination with serological or biochemical identification techniques. Results may require several days or even weeks to obtain, and may not always be ambiguous as the phenotypic characteristics by which the

bacteria are identified may be difficult to interpret and classify, or may not always be expressed [236]. Molecular techniques constitute an important advance in the detection of pathogens, offering rapid, highly specific and straightforward alternatives to traditional microbiological analyses.

The 16S rRNA gene has been widely used as a phylogenetic marker in molecular assays investigating bacterial diversity in natural ecosystems [237, 238]. It is a conserved, common but distinctive, cellular component that varies in an orderly fashion across phylogenetic lines and is present as a multicopy gene in the prokaryotic genome, making it an ideal target for taxonomic differentiation and therefore, highly specific detection, of a target microbial species [238-240]. Consequently, it has been employed widely in detection and identification of bacterial species [237, 238, 241, 242], and represents an excellent candidate for use in molecular pathogen detection assays.

Among the numerous molecular techniques available, quantitative real-time PCR (qrtPCR) is one the most widely used techniques for detection of pathogens. The ability to monitor PCR product accumulation through the early exponential stage of amplification allows qrtPCR assays the ability to both detect and quantify target DNA. This is particularly advantageous in enabling simultaneous detection of pathogen presence and pathogen loads in samples. Consequently, qrtPCR has been applied to the diagnosis of a wide range of pathogens from various sources including food products [243], fecal and environmental samples [244-247], and infected plant and animal tissues [248-251]. This includes assays developed to detect *Y. ruckeri* [252-255], though commercial applicability of these methods and their value as a screening tool is limited as they involve invasive or destructive sampling of putatively infected fish.

Use of faecal samples represents a feasible, non-invasive means of sampling putative carrier fish for asymptomatic *Y. ruckeri* infection. A non-invasive method offers the possibility of screening each individual of a population where necessary while minimising sampling impacts on stock. Such sampling strategies would offer a far more accurate understanding of asymptomatic infection

prevalence within populations than possible through random representative sampling. However, faeces represent complex biological samples not only due to the presence of a variety of bacteria in addition to the target species, but also due to a range of inhibitors such as complex polysaccharides, polyphenolic compounds, and food degradation products [256, 257]. The complexity of the faecal matrix impedes extraction of target material and inhibitory constituents can also hamper PCR assay efficiency if not effectively eliminated from the reaction, resulting in reduced analytical resolution and reliability [257-259]. Recommendations for obviating these issues have included the Griffiths method [260] and specialised commercial extraction kits [259].

Studies have previously attempted detection of *Y. ruckeri* in faeces, and initial efforts focused on the development of novel selective media met with some success, though the method could not successfully distinguish target bacteria from competing microbes in all cases [233]. More recently, selective enrichment culture media were developed and used successfully in conjunction with PCR for *Y. ruckeri* detection [261]. However, in relying on culture of bacteria from initial samples, these methods are intrinsically unsuitable for accurate and precise quantitative analysis.

The objective of this study was to develop a direct method for the detection of *Y. ruckeri* in organic matrices such as spleen and faeces obtained from finfish, and for the accurate quantification of pathogen presence even at low numbers, using a quantitative real time PCR assay.

5.2. Materials and Methods

5.2.1. *Yersinia ruckeri* culture

Tryptone soy broth (TSB; Oxoid, Basingstoke UK) was inoculated from frozen *Y. ruckeri* stock (serotype 01b, strain UTYR001) and incubated at 18°C for 18 h. Cells were concentrated by centrifuging at 8000 x*g* for 10 min. Recovered cells were washed twice by resuspending in 1M phosphate-buffered saline (PBS; pH 7.2) and centrifuging as before to eliminate any extraneous nucleic acid from lysed cells in culture. Washed cells were resuspended in 2 mL PBS that had been pre-cooled to 4°C to produce a stock suspension. Aliquots of 100 µL cell suspension diluted 1:100

in PBS were prepared in triplicate, and cells inactivated by addition of 0.03% (v/v) neutral buffered formalin (NBF) followed by incubation at 18°C for 2 h, for bacterial enumeration. Following inactivation, cell concentration in aliquots was enumerated optically using a haemocytometer and used to determine mean cell concentration in stock suspension. Stock cell suspension was diluted accordingly in PBS to achieve a final concentration of 1 x 10^6 cells mL^{-1}.

5.2.2. Organic matrices: spleen and faeces

All organic matrix samples were obtained from mortalities unrelated to this study, obviating the need for animal ethics considerations. Spleens were obtained from barramundi (*Lates calcarifer*) fry mortalities weighing approximately 13 g, which had been maintained in a pathogen-free environment and were *Y. ruckeri*-infection free. Spleens were excised aseptically, fixed in 1.5 mL RNA preservation solution (4M ammonium sulfate, 25 mM sodium citrate, 10mM EDTA, pH 5.2) over 24 h at 18°C, and then stored at -20°C. Faeces for assay validation were obtained from a fresh Atlantic salmon (*Salmo salar* L.) mortality weighing approximately 1.1 kg. To eliminate possible environmental contamination during assay validation, the entire intestine was excised, cut open longitudinally, and faeces gently scraped off and collected aseptically. Faeces were centrifuged at 100x*g* for 10 min and supernatant discarded. Concentrated faecal solids were combined with an equal weight of PBS, agitated vigorously, and used immediately in analysis.

5.2.3. *Yersinia ruckeri* dilution series and spiked sample preparation

A 10-fold serial dilution of the *Y. ruckeri* cell suspension in PBS was prepared, ranging from 1 x 10^6 cells mL^{-1} to 1 x 10^0 cells mL^{-1}. From each dilution, five sets of duplicate 1 mL aliquots were prepared, producing five series of seven duplicated 10-fold dilutions. Two of these dilution series were used to spike into faecal samples, one was used to spike spleen samples, and two maintained as pure-culture dilutions for use as standards (Fig. 5.1). In addition to molecular analysis, spiked faeces and pure-culture standards were analysed using culture-based techniques to provide comparison of assay performance.

Y. ruckeri serial dilution aliquots were combined with equal volumes of faeces suspension (≈500 mg faecal solids), producing two series of seven duplicated 10-fold serial dilutions of *Y. ruckeri* cells in faeces suspension. Spiked samples were mixed thoroughly and incubated at 4°C for 12 h, with 30 s vortex-agitation every hour. Following incubation, as per a modified technique from Rodrigues-Szulc *et al.* [262] each sample was vortex-agitated for 30 s following addition of 0.1% (v/v) Tween 20 (Sigma-Aldrich), faecal solids precipitated at 100x*g* for five min and supernatant transferred to new 1.7 mL tubes. One replicate series was analysed through culture-based methods, while the other was prepared for assessment by molecular techniques. For molecular analysis, bacteria and any remaining solids were precipitated at 8000x*g* for 10 min. Pellet and bottom 100 µL of supernatant was retained for analysis, with remaining supernatant discarded. For microbiological analysis, supernatant was used as obtained.

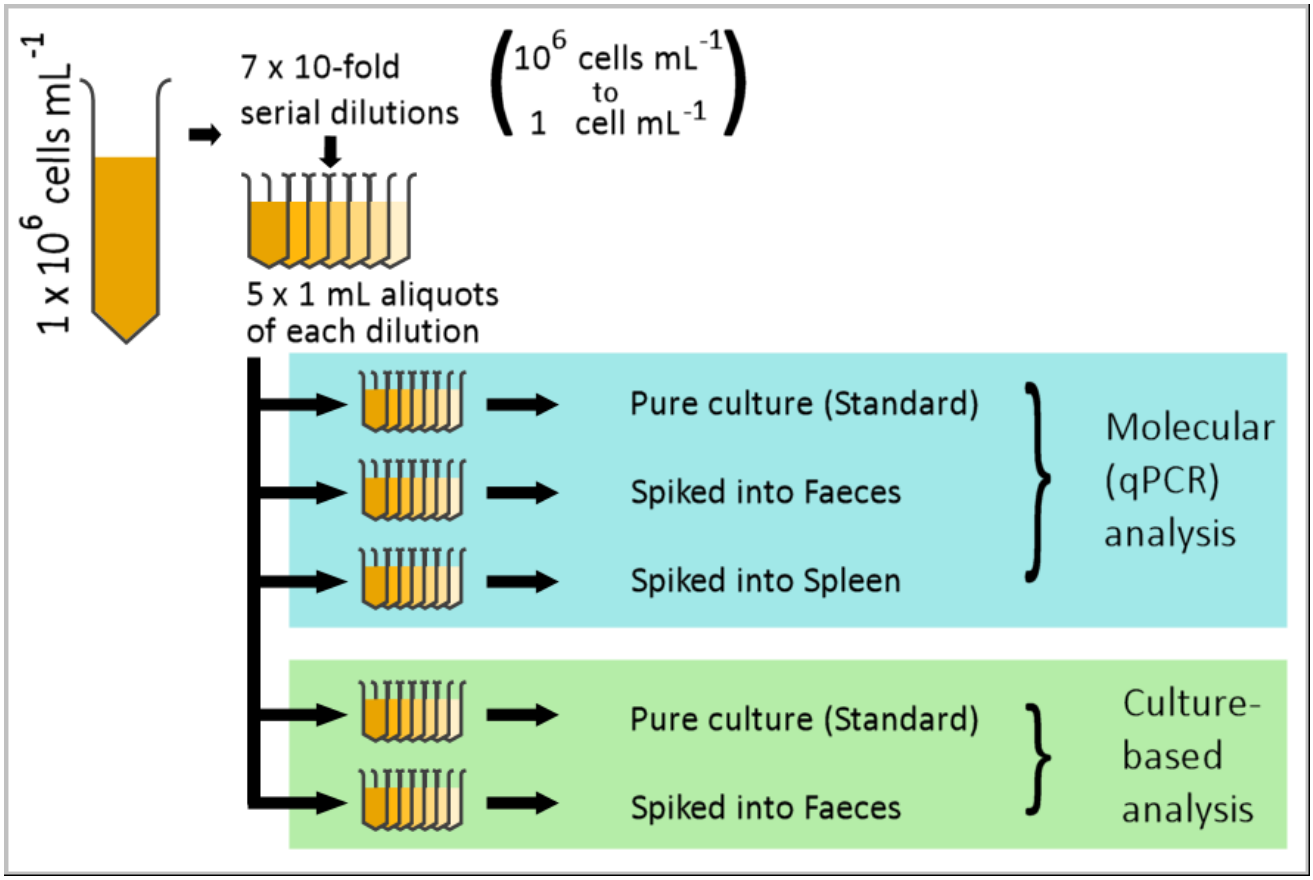

Figure 5.1: Preparation of decimal diluted *Y. ruckeri*-spiked faeces and spleen, and pure-culture standards for molecular and microbiological detection and quantification assays

Spleens (Mean weight: 25.2 ± 3.1 mg) were removed from fixative and rinsed lightly in PBS to remove excess. Individual spleens were placed in 14 separate 2.0 mL tubes containing 200 µL PBS, and homogenised aseptically using a pestle. From each serial dilution of *Y. ruckeri* 1 mL volumes were added to two spleen samples, producing seven duplicated 10-fold serial dilutions of *Y. ruckeri* cells combined with homogenised spleen. Aliquots were thoroughly mixed and incubated at 4°C for 12 h, with 30 s vortex-agitation every hour. Following incubation, bacteria and splenic solids were precipitated at 8000x*g* for 10 min. Pellet and bottom 100 µL of supernatant was retained for analysis, with remaining supernatant discarded.

One pure-culture (standard) dilution series was prepared for molecular analysis. Briefly, bacteria were harvested by centrifuging at 8000x*g* for 10 min, and pellet and partial supernatant retained as with other samples. The second standard dilution series was used for microbiological analysis without further modification.

5.2.4. Detection and quantification of *Y. ruckeri* in faeces using conventional microbiological techniques

For detection and quantification of *Y. ruckeri* in samples, supernatant from spiked faeces was applied to blood agar plates (Remel) for enumeration of colony forming units (CFU) using a simultaneous colony counting technique modified from Chen *et al.* [167]. Briefly, from each replicate of every dilution, three 10 µL volumes were applied, with a maximum of four dilutions per plate. Plates were prepared in triplicate, dried in laminar air flow for 15 min and incubated at 18°C for 24 h. *Y. ruckeri* dilutions prepared as standards (only PBS, no organic matrix) were also similarly plated and incubated. CFU were identified post-incubation based on colony morphology [233, 263] and optically enumerated to determine *Y. ruckeri* presence.

Limit of detection (LOD) was determined to be the minimum cell concentration of samples at which *Y. ruckeri* presence was detected in at least 95% of replicates.

5.2.5. Detection and quantification of *Y. ruckeri* in faeces and spleen using quantitative real-time polymerase chain reaction (qrtPCR)

5.2.5.1. Extraction of total nucleic acid

All samples were incubated at 37°C for 30 min after the addition of 395 µL extraction buffer (4 M Urea, 0.2 M sodium choride, 1 mM sodium citrate, 1% SDS) supplemented with 5 µL Proteinase K to lyse cells. The resulting suspension was cooled on ice for 5 min and protein, cellular debris, and detergent were removed by centrifugation in 7.5 M ammonium acetate at 14000x*g* for 5 min at 18°C. Nucleic acids were recovered by isopropanol precipitation at 14000x*g* for 10 min at room temperature. The nucleic acid pellet was washed twice with ethanol and eluted in 200 µL elution buffer [molecular-grade water containing 10 µM TRIS-HCL and 0.05% (v/v) Triton X100 (Sigma-Aldrich)].

5.2.5.2. Quantitative real-time PCR

All qrtPCR analyses were conducted on a CFX Connect Real-Time PCR detection system (Bio-Rad) with efficiency and stringency of standard curves held to between 85-110% and 0.98-1.00. A TaqMan probe-based PCR assay was developed for detection of *Y. ruckeri.* Primers used to amplify a 247-bp region of the *Y. ruckeri* 16S rRNA gene were developed and tested exhaustively to establish intra- and inter-species specificity in previously published research [264]. Primer and probe sequences used are presented in Table 1. Each PCR reaction consisted of 5 µL 2X MyTaq HS Mix (Bioline), forward and reverse primers (400 nM each), *Y. ruckeri* 16S ribosomal gene-specific TaqMan probe (100 nM) and 2 µL template in molecular grade water to a final volume of 10 µL. No-template controls (NTC) using molecular grade water instead of template, extraction controls and sample controls (unspiked) were included in each run. Cycling conditions consisted of an initial activation of DNA polymerase at 95°C for 3 min, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. Assay results were quantified by analysis of raw fluorescent unit (RFU) data from cycles 5 to 40 using the CM3 mechanistic model developed by Carr and Moore [265] included in the qPCR package (v. 1.4-0; [266]) for RStudio statistical computing software [267], and verified empirically.

Table 5.1: Primers and 16S rRNA gene TaqMan probe used for *Y. ruckeri* detection and quantification by qrtPCR

Forward primer [263]	AAC CCA GAT GGG ATT AGC TAG TAA
Reverse primer [263]	GTT CAG TGC TAT TAA CAC TTA ACC C
Probe (TaqMan)	AGC CAC ACT GGA ACT GAG ACA CGG TCC

5.2.5.3. Establishment of Limit of Detection (LOD) and Limit of Quantification (LOQ)

Based on 16S gene abundance in various strains of three species of the genus *Yersinia*, as available on the Ribosomal RNA Database (v4.3.3 [268]), *Y. ruckeri* was estimated to have between 6 and 7 16S rDNA gene copies per cell.

Limit of detection (LOD) for the qrtPCR assay used was determined to be the minimum concentration at which the target gene fragment could be amplified with 95% certainty. Limit of quantification was defined as the minimum concentration at which the assay accurately identified an expected number of 16S gene copies in 100% of samples with a mean coefficient of variation (CV) of less than 35%.

For LOD and LOQ determination 100 µL aliquots of eluted nucleic acid were pooled from each replicate of the highest concentration *Y. ruckeri*-PBS samples. Pooled nucleic acid suspension was diluted in elution buffer to a concentration equivalent to approximately 5×10^3 16S gene copies mL^{-1}. From this suspension, 16 independently diluted replicates of five different dilutions (5×10^3, 4×10^3, 2×10^3, 5×10^2, and 2.5×10^2 16S gene copies mL^{-1}) were prepared.

Replicate dilutions were used at 2 µL $reaction^{-1}$ in qrtPCR, producing mean estimated copy-concentrations of 8, 4, 1 and 0.5 copy $reaction^{-1}$. Assay conditions, and reagent concentrations were identical to those previously used. Results were quantified by analysing RFU data from cycles 5 to 40 as before, and compared to expected copy number distributions determined using the LRE Analyzer tool [269].

5.3. Results

5.3.1. Microbiological detection and quantification of *Y. ruckeri* in spiked faeces

No CFU were detected in dilutions corresponding to fewer than 100 cells mL-^{1} using culture-based methods in either faecal samples or standards (pure culture). The LOD of the assay, where at least 95% of replicates showed presence of *Y. ruckeri*, was ≥ 1 x 10^3 cells mL^{-1}. The LOQ of the assay was determined to be 1000 cells mL^{-1}. Mean concentration of *Y. ruckeri* was 939 cells mL^{-1} (CV=0.04%) for standards and 1022 cells mL^{-1} (CV=0.08%) for spiked faecal samples.

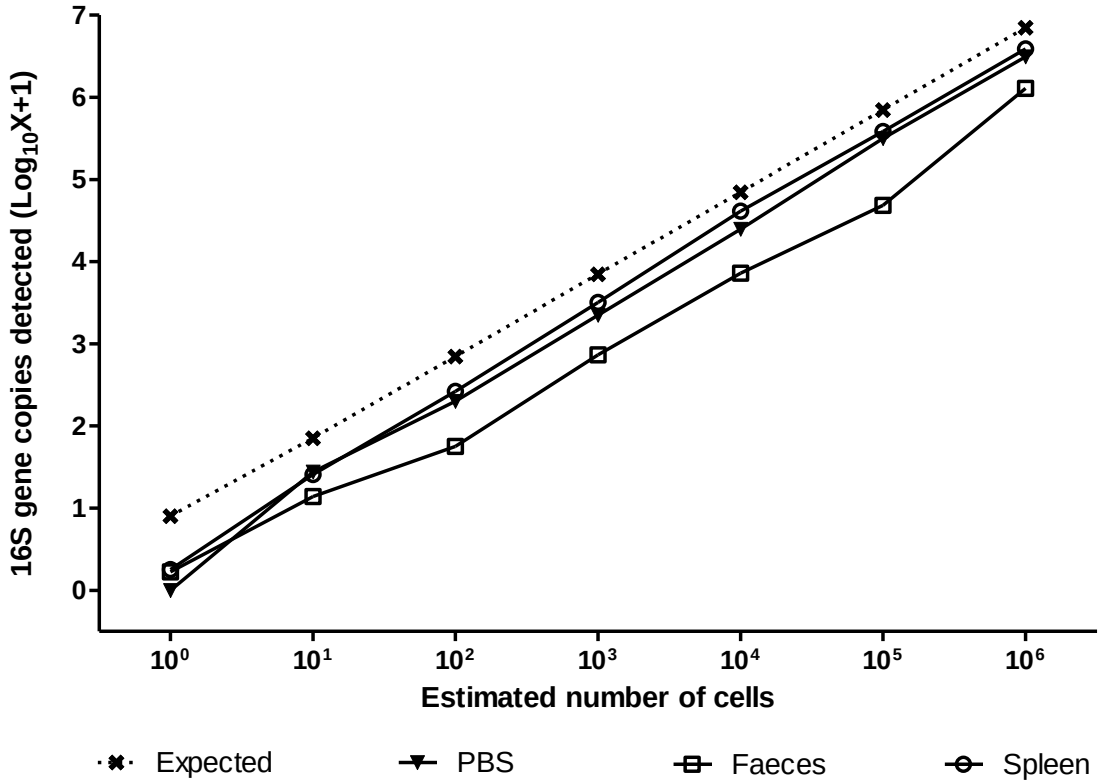

Figure 5.2: *Y. ruckeri* 16S gene copies detected from serial 10-fold dilutions of *Y. ruckeri* cell suspension in PBS and from spleen and faecal samples spiked with identical serial dilutions, in comparison with expected number of copies based on an estimated seven 16S gene copies per cell.

5.3.2. Detection and quantification of *Y. ruckeri* in faeces and spleen using qrtPCR

There was no amplification in either NTCs or extraction controls. Samples were considered *Y. ruckeri*-positive when at least one of two replicate wells was successfully amplified. *Y. ruckeri* was successfully detected in all samples analysed by qrtPCR except the lowest concentration (10^0 cells mL^{-1}) in standards, demonstrating linearity from 10^1 to 10^6 cells mL^{-1} (Efficiency=99.9%;

R^2=0.998; Slope=3.325). Amplification in all spiked faecal samples was delayed in comparison with standards and spiked-spleen samples, exhibiting approximately 4-fold loss in sensitivity that resulted in *Y. ruckeri* being detected at lower levels than corresponding standards (Fig. 5.2).

5.3.3. Limit of Detection (LOD) and Limit of Quantification (LOQ) for qrtPCR assay

Limit of Detection for the assay was established at ≥ 4 copies, at which dilution 100% of replicates were amplified successfully. Limit of Quantification was determined to be 10 copies, at which dilution mean detected copy number was 7.37 (CV=31.54%).

5.4. Discussion

Successful detection and absolute quantification of *Yersinia ruckeri* in barramundi spleen and Atlantic salmon faeces was achieved in this study using qrtPCR-based detection of 16S ribosomal DNA. The methods presented here provide an effective alternative to conventional microbiological methods for detection and quantification of *Y. ruckeri*, offering rapid and highly sensitive analytical ability over a 6-logwide linear dynamic range using non-invasively acquired samples.

The assay developed here was able to detect and quantify low levels of *Y. ruckeri* in spiked faecal samples, supporting the viability of this method of faecal testing as a straightforward, non-invasive strategy for screening fish populations. For the purposes of assay validation, faeces were obtained from excised intestine to minimise environmental contamination. However, aseptic collection of manually stripped faeces, preceded by topical decontamination of the vent area, would be sufficient for field applications. Analytical success achieved here using samples from different finfish species also suggests broad applicability of the method. The molecular approach used provides a distinct advantage in specificity and speed of analysis compared to outcomes from culture-based methods, which typically take a minimum of 2 to 3 days to achieve. In contrast, the qrtPCR assay developed here can provide highly specific, absolute quantification of *Y. ruckeri* presence in infected samples within approximately 2.5 h. Commercial multisubstrate identification systems commonly used in culture-based detection may result in multiple possible result profiles due to variable expression or

masking of bacterial phenotypic characteristics, often making interpretation subjective and therefore of limited reliability [233, 270]. PCR methods provide considerably greater specificity, and PCR assays have previously been developed successfully for detection of *Y. ruckeri*. However, these assays were reliant on analysis of blood or organs obtained through invasive or lethal sampling of potentially infected fish [252-255, 271]. The potential negative impact of such sampling methods on stock make these strategies less feasible as screening tools than the method developed in this study.

The lower limit of detection in previous studies using PCR and qPCR assays to detect *Y. ruckeri* in fish tissues ranged from 3.4 to $2x10^4$ CFU g^{-1} [253, 254, 272, 273]. In comparison, the assay developed here demonstrated greater sensitivity (0.5 CFU g^{-1}) even when analysing faecal samples, in spite of the difficulties associated with molecular analysis of faeces [257]. Sensitivity of the current method was comparable to the most sensitive assays used in other studies developed for bacterial detection in faecal samples, where lower limits of detection have ranged from 10 to $1x10^4$ copies of the target gene sequence [274-279]. Analysis of faeces spiked with serial decimal dilutions of *Y. ruckeri* cells demonstrated this assay could detect single-cell amounts of *Y. ruckeri*, equivalent to approximately 7 copies of the target gene sequence, in 500 mg of suspended faecal solids or in 25 mg of splenic tissue.

The sensitivity of qrtPCR may be affected by a variety of factors, including the quantity and quality of the extracted nucleic acid, and the presence of inhibitory substances [258]. The reliability of a qrtPCR assay must therefore be assessed in the context of its lower limit of detection (LOD) and limit of quantification (LOQ) [280]. The detection limit of this assay (LOD) was 4 copies of the target gene sequence and the LOQ was determined to be 10 copies. Conservatively estimating 7 copies of the 16S gene per cell based on other *Yersinia* species [268], the LOQ and LOD of the assay were approximately 1 cell and 0.5 cell respectively, or the equivalent of 2.65 ag and 1.07 ag of the target gene transcript respectively, per qrtPCR reaction. In comparison, using bacterial culture-based methods, the LOQ and LOD were both 10^3 CFU mL^{-1}, indicating the deficiency in

sensitivity compared to the qrtPCR assay developed here and rendering further detailed investigation, such as accurate identification and quantification of all isolated bacteria, superfluous in the context of this study.

To address analytical difficulties due to faecal matrix complexity in this study, protocols for extraction of bacteria from food matrices were adapted to minimise faecal pathogen retention through the addition of Tween 20, a polysorbate surfactant [262]. This was supplemented with a nucleic acid extraction protocol optimised empirically for use in this assay. Some PCR inhibition was apparent in faecal samples examined in this study in spite of the optimisation measures used. Problems associated with molecular detection of pathogens in faecal samples from finfish have previously been addressed through the inclusion of a selective enrichment culture step designed to differentially increase target abundance [233, 281]. However, this compromises accuracy and quantitative precision due to non-uniform replication of bacterial cells within a sample. Target distribution is heterogeneous at low concentrations in samples and does not conform to stochastic variation. Cells may also be non-culturable in spite of being viable [236]. As a result, there is an elevated possibility for Type II error using such methods, as demonstrated by the false negative results obtained at low dilutions through culture-based methods in this study. In contrast, the outcomes from the qrtPCR assay were consistent and capable of reliably detecting single-cell quantities of *Y. ruckeri*. Further optimisation of the extraction protocol, possibly employing methods such as those demonstrated by Schunck *et al.* [282], could reduce PCR inhibition and improve the current assay.

Quantification of amplified product in qrtPCR has traditionally been achieved by comparison of relative amplification with a standard of known starting target quantity. The inherent difficulty in constructing target-specific standard curves that are consistent across multiple assays has impeded broad adoption of absolute quantification, particularly for large-scale applications [283]. A primary assumption in standard analysis of qrtPCR data is that of equal amplification efficiency, with any

variation from this being interpreted as a variation in amplifiable template quantity, as opposed to intrinsic variation due to differences in thermal cycler performance or reagent formulations [284]. In an attempt to account for intrinsic variation, studies have included coamplified internal controls in qrtPCR assays. However, evidence suggests that preferential amplification of one template over another may be common in such a scenario [285-287]. Absolute quantification obviates the need for constructing standards as well as issues arising from intrinsic variations in amplification efficiency by applying a mechanistic model that accurately predicts product accumulation of through an entire reaction and thereby determines initial template quantities [265, 288]. In this study, a mechanistic model developed by Carr and Moore [265] was used to quantify detected amounts of the *Y. ruckeri* 16S rDNA gene transcript.

At low template concentrations, sources of intrinsic variation and chemical factors from complex host matrices can combine to also introduce error in a quantitative PCR assay due to significant differences in amplification efficiencies during the cycles of the amplification process [284, 289]. To account for this, the first four cycles of the qrtPCR assay in this study were excluded from analysis. An additional consideration at low template concentrations is the intrinsic variation in target quantity between samples at very low copy numbers, which does not conform to stochastic distributions but can be modelled using a Poisson distribution [280, 288]. This explains the difference between LOD and LOQ observed for the qrtPCR assay developed here. However, LOD in this assay was close to the stochastic qPCR sensitivity limit of 3 copies, which assumes a Poisson distribution, a 95% chance of including at least 1 copy in the reaction, and the ability to detect single copies [290]. Attempting to reliably detect lower concentrations would extend into digital PCR strategies that are not practicable for the intended scope and application of this assay [280, 289].

The ubiquity of *Y. ruckeri* and its ability to establish and maintain asymptomatic infection in fish make it a particularly problematic threat to finfish aquaculture. This study provides the ability to

detect and quantify infection *Y. ruckeri*, even at low levels, and without the need for invasive or lethal sampling, which will of benefit to the aquaculture industry. With further optimisation of extraction protocols to minimise inhibition, the qrtPCR assay developed presents an effective screening tool for future epidemiological surveys. It could potentially form an important part of aquaculture disease management strategies targeting *Y. ruckeri*, especially in the context of investigating asymptomatic infection.

CHAPTER SIX

Oral immunisation for protection of first-feeding Atlantic salmon, *Salmo salar* L., against yersiniosis

6.1. Introduction

Early investigations demonstrated the efficacy of immersion vaccination of fish in a bacterin as a successful strategy for protecting farmed salmonids against *Y. ruckeri*, and an immersion vaccine consisting of formalin-inactivated whole cells was commercially licensed in 1976 in the USA [291]. A similar vaccine, developed by DPIPWE Launceston, Tasmania, has been used extensively to vaccinate fingerlings (body weight 5 g) in the Australian salmonid industry. However, outbreaks still occur, and mortality of approximately 500,000 fish occurred over a six-month period in 2007 despite stocks having been vaccinated [292]. *Yersinia ruckeri* is capable of establishing and maintaining subclinical infection, resulting in asymptomatic carriers, which may be responsible for such outbreaks [235]. Current fish health management strategies have been unsuccessful in preventing the development of subclinical *Y. ruckeri* infection, and an immunoprophylaxis strategy capable of inhibiting establishment of asymptomatic carriers would therefore prove extremely beneficial for salmon health management [232, 234, 235].

At present, salmonid fry are initially immunised against *Y. ruckeri* by immersion vaccination at approximately 2 – 5 g size [293, 294], at which point they are still too small for intraperitoneal vaccination but large enough to handle without major deleterious impacts. Before reaching this size, S. salar fry are not considered to have developed sufficient adaptive immunocompetence, and are typically not provided with any form of immunoprophylaxis. Given the ubiquity of *Y. ruckeri*, and its ability to survive in the environment without a host [295, 296], the risk of infection in small fish is considerable. Immune system components generally develop early in freshwater fish [297], and recently, bacterial challenge of rainbow trout larvae and fry has shown that first-feeding salmonids may possess a range of innate immune factors that offer protection from infection, though the mechanisms involved are not clear [298]. These findings suggest that enhancing the immune response in Atlantic salmon fry against pathogenic infection may be possible. Recently, onset of *Y. ruckeri* infection has been observed in fish smaller than the minimum vaccinated size in commercial

Atlantic salmon populations, indicating the importance of developing an effective means of protecting smaller fish that is also practicable on a commercial scale.

Mucosal administration of antigens offers the most feasible approach for immunisation of small fish. Mucosal immunisation also specifically targets stimulation of mucosal immunity in the fish, which arguably provides the first line of defense against most pathogens that fish are exposed to [23, 38]. Oral immunoprophylaxis in particular represents an ideal strategy for this purpose as it has no fish-size limitations and requires minimal infrastructure and specialized skills for effective implementation. However, protection conferred by oral immunisation has proved inconsistent in trials [179-183]. Digestive degradation has been implicated as the major cause of this inconsistency, as antigenic integrity must be retained until the immunogen reaches the distal intestine, which has been identified as an immunologically active part of the gastrointestinal tract involved with uptake of antigens [34, 39, 299]. Some studies in different teleost species have also reported observations of oral tolerance, a phenomenon characterized by a decrease in immune response linked to the extended administration of orally delivered antigens in various fish species including salmonids [205-207], suggesting interrupted administration regimes to address the issue.

Biopolymeric microencapsulation of orally administered antigens has demonstrated some success in protecting fish from pathogens. Besides effectively protecting immunogenic material from digestive degradation, microencapsulation increases its bioavailability due to particulate dispersion, facilitates antigen uptake in the distal intestine and offers the possibility of affecting controlled release of the antigenic substance. Several biopolymers used in antigen microencapsulation are also known to have intrinsic adjuvant properties, making their use advantageous in immunoprophylaxis [300, 301].

The aim of this study was to assess protection afforded to first feeding Atlantic salmon fry against bacterial challenge with pathogenic *Y. ruckeri* when immunised by using a microencapsulated oral

Y. ruckeri vaccine, and assess whether this immunisation strategy affected establishment of asymptomatic carriers.

6.2. Materials and Methods

6.2.1. Ethics statement

All procedures on fish were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and approved animal handling guidelines (University of Tasmania Animal Ethics Committee approval Ref: A12285).

6.2.2. Fish source, maintenance and experimental design

Atlantic salmon (*Salmo salar* L.) were used throughout this study. For *in vivo* uptake investigation, 20 fry with a mean weight of 1 g were obtained from a commercial Tasmanian hatchery and held in a 20 L mesh enclosure within a 1000 L tank supplied by recirculating freshwater at 10°C. For immunisation studies, pathogen-free alevin were obtained from a commercial Tasmanian hatchery prior to commencement of exogenous feeding. They were acclimated for three weeks in a UV-sterilised freshwater recirculating system at 10°C. Post-acclimation, fish at a mean weight of 0.32 g were transferred to 200 L tanks, supplied by UV-sterilized recirculating freshwater at 10°C. Other water quality parameters including pH (7.2–7.6), ammonia (<0.25 mg L^{-1}), nitrite (<0.25 mg L^{-1}), nitrate (<0.5 mg L^{-1}) were monitored daily. Exogenous feeding commenced 24 h after transfer, and fish were then fed daily to satiation with a commercial crumble feed (Skretting, Cambridge, Tasmania). Tanks were randomly allocated to treatment groups, comprising three replicate tanks containing 35 fish each (Table 1). Fish were starved for 24 h seven days after initial feeding, following which experimental treatment administration was commenced.

6.2.3. *Yersinia ruckeri* culture

Yersinia ruckeri (serotype 01b, strain UTYR001) was grown as described in Chapter 5 to produce a 5 mL starter culture. This was used to inoculate 50 mL TSB (1:100 v/v), which was incubated at 18°C overnight, and in turn used to inoculate 5 L TSB (1:100 v/v), and incubated at 18°C for 24 h

with constant aeration for use in immunoprophylactic preparation. For bacterial challenge, the same procedure was followed to achieve a final culture volume of 7 L.

6.2.4. Preparation of oral vaccine

Cultured *Y. ruckeri* cells were inactivated by the addition of neutral-buffered formalin at 0.3% of total volume and subjecting the culture to constant agitation over 24 h. TSA plates were inoculated with 100 µL of inactivated bacteria in triplicate, and incubated at 18°C for 24 h to confirm bacterial inactivation. Inactivated bacteria were concentrated by centrifuging at 8000x*g* for 30 min and washed twice in PBS. Concentrated cells were combined with distilled water (4:1 v/v) and resuspended by 60 s vigorous agitation. Cells in suspension were disrupted by four cycles of ultrasonication (60 s on / 30 s off) while held on ice.

Y. ruckeri lysate was microencapsulated using methods adapted from Chapter 2. Briefly, lysate was combined with a 4% (w/w) aqueous solution of sodium alginate salt (medium viscosity; Sigma-Aldrich) in 1:3 ratio (v/v) and stirred for 10 min to produce a 3% final alginate concentration. This was gradually introduced into the oil phase (octane containing 7.5% v/v Span-80; Sigma-Aldrich) in a 1:2 ratio (v/v) and emulsified by stirring at approximately 8000 rpm with the addition of Tween-80 (3.3% v/v; Sigma-Aldrich). Microcapsules were calcium-crosslinked over 45 min, hardened in isopropyl alcohol for 25 min and recovered by centrifuging the mixture at 2000x*g* for 10 min. Recovered microcapsules were washed twice in distilled water and then lyophilised.

Commercial feed was lyophilised until reduced in weight by 50% and crushed to a fine powder. Lyophilized microcapsules were mixed with powdered feed (1:100 w/w) until a uniform mixture was achieved. Distilled water was added to the mixture (50% w/w) and combined to form a dry paste, which was extruded and dried at 18°C. Dried treated feed was crushed coarsely and sieve-separated to obtain particles commensurate to fish size over the duration of treatment administration.

6.2.4.1. Fluorescent labelling of vaccine microcapsules

All protocols involving fluorescein isothiocyanate (FITC; Sigma-Aldrich, St. Louis MO, USA) were performed under protection from light exposure. *Y. ruckeri* microcapsules were labelled for validation of uptake after oral administration by affecting the modifications to the oral vaccine manufacture process.

Briefly, sonicated *Y. ruckeri* lysate was fluorescently labelled by combining with FITC solution (2.5% w/w in 1M phosphate-buffered saline; PBS) at a 2:1 ratio (v/v) and incubated at 30°C for 4 h. FITC-labelled cell lysate was dialysed against 0.01M PBS over 24 h to remove superfluous FITC. To produce FITC-labelled alginate, FITC solution (in 1M PBS) was combined (0.15% v/v) with 4% (w/w) aqueous alginate solution previously adjusted to pH 9, and incubated for 1.5 h at 40°C with continuous stirring. FITC-labelled alginate solution was dialysed against distilled water for 24 h at 4°C to remove any uncoupled FITC. FITC-labelled lysate was combined with FITC-labelled alginate solution in 1:3 ratio (v/v) and stirred for 10 min to produce a 3% final alginate concentration, which was used as the aqueous phase for manufacture of FITC-labelled microcapsules, subsequently combined combine with feed as described.

6.2.5. Preparation of immersion vaccine

Vaccine for immersion immunisation was prepared using a 24 h culture of *Y. ruckeri* cells in TSB, grown with constant aeration at 18˚C. Culture was inactivated by the addition of neutral-buffered formalin at 0.3% of total volume followed by constant agitation over 24 h, and then stored at 4°C until used for immunisation. TSA plates were inoculated with 100 µL of inactivated bacteria in triplicate, and incubated at 18°C for 24 h to confirm bacterial inactivation. Inactivated culture was diluted 1:10 for immunisation, to achieve a final suspension of approximately 1.38×10^9 cells mL^{-1}.

6.2.6. Immunisation

Fish were divided into three treatment groups comprising an untreated control group (CONTROL), fish receiving orally administered vaccine (ORAL) and fish that received an orally administered

vaccine as well as a booster immersion-vaccination (ORAL+DIP). Treatments for groups are summarised in Table 6.1.

Prior to commencement of oral immunisation treatments, all fish were fed untreated commercial feed *ad libitum* for 14 days after treatment-group allocation, during which time palatability of treated feed was assessed using 10 fish maintained in an isolated system. Vaccine-treated feed was administered according to a staggered regime to minimise potential development of oral tolerance (see Rombout *et al.* [22]). While fish in the untreated CONTROL group were maintained on untreated commercial feed throughout, ORAL and ORAL+DIP groups received treated feed, prepared as described, for seven consecutive days, followed by seven days of untreated feed. This 14-day regime was repeated thrice, achieving 21 days of treated feed administration in total. Following completion of the oral treatment regime, all fish were returned to untreated commercial feed until challenge.

When at a minimum weight of 1 g (Mean weight 1.78 g), feed was withheld from fish in the ORAL+DIP group for 24 h, following which they were administered a booster immunisation via immersion. Fish from each replicate tank were immersed in 5 L of previously prepared vaccine suspension for 60 s under constant aeration, followed by transfer to running dechlorinated freshwater for 60 s before being returned to their respective tanks. Feeding with untreated commercial feed was resumed 24 h after booster immunisation.

Table 6.1: Treatment groups and vaccination regime for immunisation of first-feeding Atlantic salmon fry against *Y. ruckeri*

Group Label	**Immunisation**	**Fish/Tank (3 tanks/treatment)**
ORAL	3 x (7 d treated feed / 7 d untreated feed)	20 (+ 15 for sampling)
ORAL+DIP	3 x (7 d treated feed / 7 d untreated feed) + Booster Immersion (Mean Wt. 1.78 g)	20 (+ 15 for sampling)
CONTROL	No treatment	20 (+ 15 for sampling)

6.2.7. Sampling

6.2.7.1. Establishment of *Y. ruckeri*-free status

Immediately after transfer to acclimation tanks, 10 randomly selected individuals (approximately 0.32 g body weight) were lethally anaesthetised (5 ml L^{-1} Aqui-S NZ, Lower Hutt, New Zealand). Each fish was rinsed thoroughly to remove traces of anaesthetic, homogenised in 1 mL PBS, and the homogenate was incubated for 24 h at 18°C on TSA plates for analysis of colonies using colony morphology and PCR to confirm *Y. ruckeri*-free status.

6.2.7.2. Oral uptake validation

To determine uptake of oral vaccine, 15 fry were randomly selected prior to group allocation and transferred to three enclosures (five fish per enclosure) in an isolated system with identical environmental parameters and maintained on untreated commercial feed administered *ad libitum* until approximately 1 g (Mean weight 1.3 g). For fish in two of the enclosure (n = 10) commercial feed administration was discontinued and feed combined with FITC-labelled *Y. ruckeri* microcapsules was administered *ad libitum* twice over a 24 h period. Fry in the third enclosure received an identical administration of commercial untreated feed. All fish were lethally anaesthetised 48 h after final feed. Maintaining protection from light exposure, spleen, liver and head kidney were removed and fixed in Davidson's (freshwater) fixative over 24 h, and then prepared for histology by ethanol-series dehydration, paraffin infiltration and embedding in paraffin blocks. The blocks were sectioned at 5 µm using a microtome (Microm HM340, Germany) and mounted on glass slides, all according to standard histological procedures. The sections were observed under a compound microscope equipped with fluorescent illumination (Olympus BH2, Japan) using objective magnifications of 10X and 20X, and uptake of FITC-labelled microcapsules and contents was evaluated visually.

6.2.7.3. Challenge mortality

Throughout the challenge period, reisolation of *Y. ruckeri* was attempted from 20% of daily mortalities per tank by inoculating TSA plates with head kidney samples excised from mortalities.

Colonies were identified using *Y. ruckeri*-specific 16S ribosomal gene primers through PCR to confirm *Y. ruckeri* as the cause of mortality.

6.2.8. *Y. ruckeri* challenge

Six randomly selected fish from each tank were transferred to a pathogen-free system with identical environmental conditions and water supply (n=18 per group) as challenge controls (mock infected). Nine weeks (at 10°C) after administration of booster immunisation, fish from all three replicate tanks within each treatment group were challenged by a 60 min immersion in 15 L freshwater at 10°C, saturated with air and containing pathogenic *Y. ruckeri* (75 mL culture) at a final concentration of 3.8 x 10^7 colony forming units (CFU) mL^{-1}. Initially estimated by optical enumeration, the dose was confirmed as per previously published methods [167] using TSA plates incubated at 18°C for 36 h. Following immersion, fish were returned to their respective tanks. Tanks were monitored for mortalities, which were sampled as described, for 21 days post-challenge. Cumulative percent mortality (CPM) from each treatment was used to calculate the relative percent mortality (RPS) as RPS = (1 – (mean treatment group CPM/control group CPM)) x 100. Challenge control (mock infected) fish were mock-challenged by similar immersion in 15 L freshwater containing 75 mL sterile TSB before being returned to their respective enclosures.

6.2.9. Asymptomatic carrier analysis

Following termination of challenge, challenge survivors were transferred from challenge tanks to one of three enclosures corresponding to each treatment group in a system free from *Y. ruckeri*. Feeding with commercial feed was resumed 24 h after transfer, and enclosures were observed for mortalities over four weeks. At the end of this period, all surviving fish from each group were lethally anaesthetised. Spleens were excised, fixed in 1.5 mL RNA preservation solution (4M Ammonium sulfate, 25 mM Sodium citrate, 10mM EDTA, pH 5.2) over 24 h at 18°C, and then stored at -20°C. Whole spleen from each fish was analysed for *Y. ruckeri* load using a real-time qPCR assay as described in Chapter 5, to determine asymptomatic carrier status of fish in each group. Spleens were rinsed in water to remove excess fixative and cut into pieces (approximately 2

mm x 2 mm) to facilitate efficient lysis. Nucleic acids were extracted and purified using a Proteinase K-supplemented Urea extraction buffer and ammonium acetate precipitation, and collected through isopropanol precipitation, as described in Chapter 5.The nucleic acid pellet was washed twice with ethanol and eluted in 100 µL water containing 10 µM TRIS-HCL and 0.05% TritonX (v/v).

6.2.10. Quantitative real-time PCR (qrtPCR) analysis

All qrtPCR analyses were conducted on a CFX Connect Real-Time PCR detection system (Bio-Rad) with efficiency and stringency of standard curves held to between 85-110% and 0.98-1.00. To minimise inhibition from the large amount of host DNA, eluted nucleic acid was diluted 1:10 in molecular grade water before analysis as per protocols developed for detection of *Y. ruckeri* in Chapter 5. Assay results were quantified by analysis of fluorescent unit (rfu) data.

6.2.11. Statistical analysis

Analysis of realtime qPCR results from the *Y. ruckeri* detection assay was performed using the cm3 model in the 'qpcR' package [266] for R. Analysis of Variance (ANOVA), performed through the 'ez' package [302] for R, was used to compare differences between treatments as appropriate, applying Levene's Test to verify homoscedasticity and with results considered significantly different at $P \leq 0.05$. Tukey's HSD Post-Hoc test was used to determine significantly different treatments. Survival curve analysis was performed using the Log-rank test in the 'survival' package for R [303], with a Bonferroni correction set to allow for multiple pairwise curve comparisons. Difference in percentage of asymptomatic carriers was tested for significance using Chi-square analysis (Mantel-Cox Log rank test).

6.3. Results

6.3.1. *In vivo* microcapsule uptake and content distribution

Distinct areas of fluorescence were observed in head kidney, liver and spleen from fish administered with fluorescently labelled vaccine-treated feed, indicating translocation of the FITC-

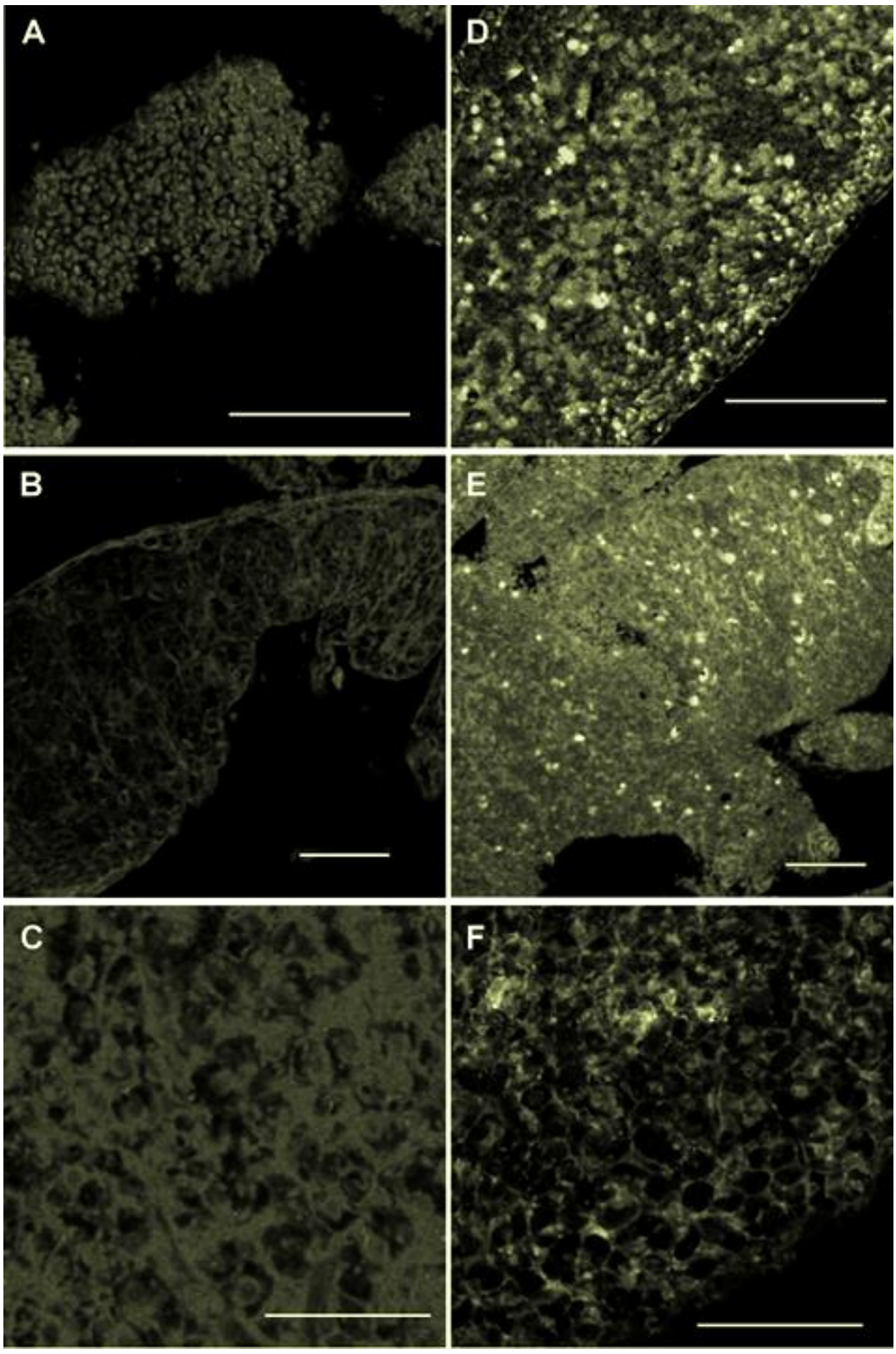

Figure 6.1: Fluorescent optical micrographs of samples from negative controls (A: Spleen, B: Head kidney, C: Liver), and from fish fed FITC-labelled vaccine-treated feed (D: Spleen, E: Head kidney, F: Liver). Bar=100µm

labelled material to immunologically important organs, and retention over 48 h post-administration (Fig. 61). No such fluorescence was observed in organs of fish fed only untreated commercial feed.

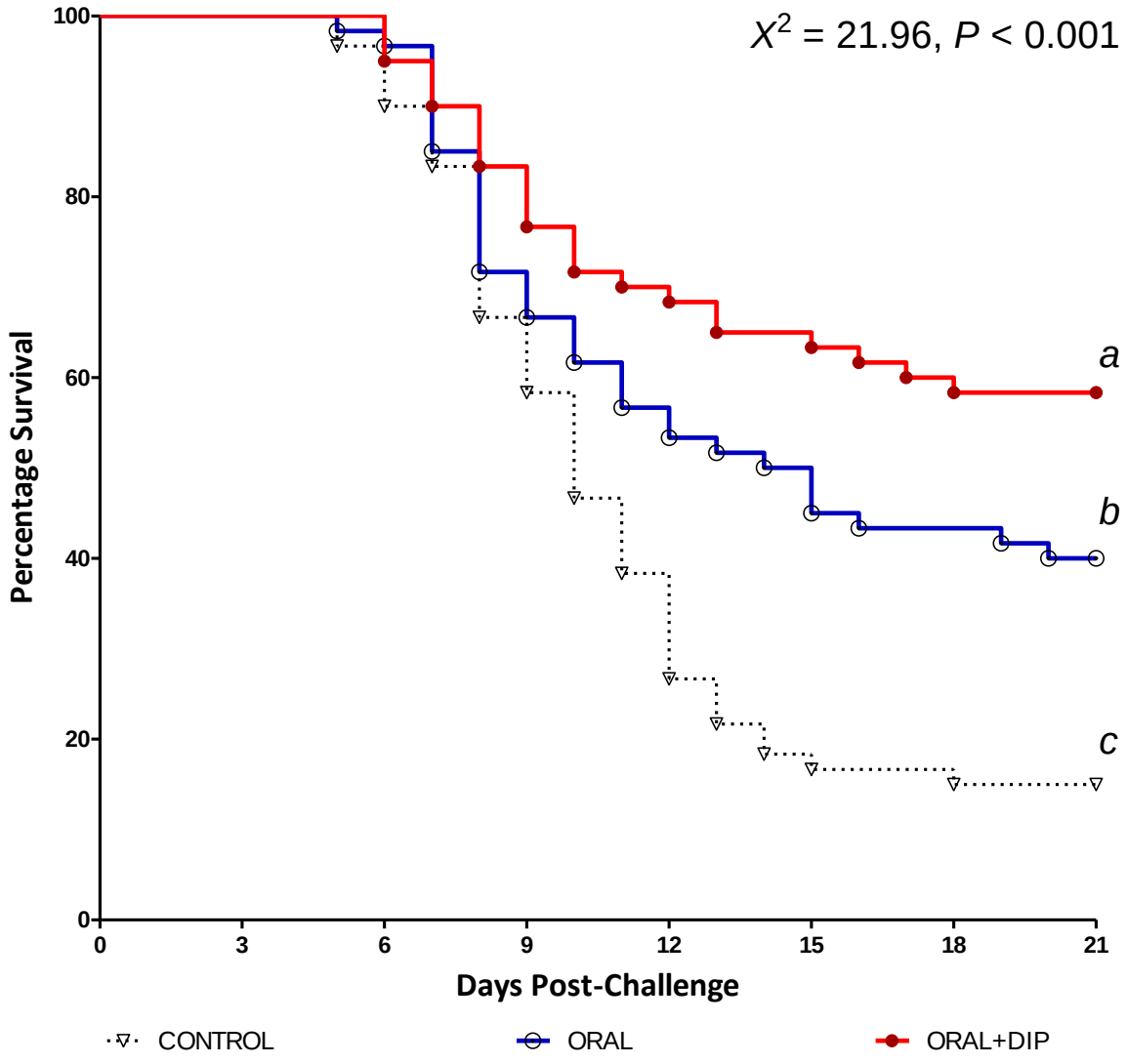

Figure 6.2: Post-challenge survival with *Y. ruckeri* in first-feeding Atlantic salmon fry immunised orally, with and without a booster immersion-immunisation at 1.0 g size. Different lowercase letters indicate significantly different treatments.

6.3.2. *Y. ruckeri* challenge

Both ORAL and ORAL+DIP groups demonstrated moderate protection against *Y. ruckeri* challenge compared with untreated controls, with RPS values of 29.4% and 51% respectively. CPM levels did not differ significantly between the ORAL (60.0 ± 0.0) and ORAL+DIP (41.7 ± 13.0) groups, though both groups had significantly lower CPM than untreated controls (F = 7.98, df = 2,6, P = 0.020). In contrast, survival curve analysis indicated a significant difference in survival kinetics between the each of the immunised groups as well as the untreated controls (X^2 = 21.96, P < 0.001) (Fig. 2). No mortalities were observed in any of the challenge control (mock infected) fish.

Table 6.2: Percentage of surviving Atlantic salmon in each treatment group identified as asymptomatic *Y. ruckeri* carriers in each group, and median splenic bacterial load (expressed as number of *Y. ruckeri* 16S ribosomal gene copies detected)

Group	**Asymptomatic carriers (% of survivor population)**	**Median Load (ribosomal 16S gene copies)**
ORAL	55.56	3.0×10^1
ORAL+DIP	51.43	1.30×10^2
CONTROL	59.09	3.23×10^1

6.3.3. Asymptomatic carrier status

None of the survivors exhibited any abnormal behaviour or gross physiological signs characteristic of yersiniosis when sampled. All groups included some asymptomatic carriers, based on analysis of survivor spleens for systemic presence of *Y. ruckeri* (Table 6.2). Percentage of asymptomatic carriers detected in each group ranged from 51.43% (ORAL+DIP) to 59.09% (CONTROL), though there were no significant differences between groups.

6.4. Discussion

The oral immunoprophylactic treatment administered to first-feeding *S. salar* fry in this study clearly conferred protection against mortality due to *Y. ruckeri* infection, as evidenced by significantly lower CPM levels than untreated controls. This study was conducted in parallel with a study examining the protective efficacy of immersion vaccination of salmon fry belonging to the same cohort prior to commencement of exogenous feeding, utilising the same control fish and *Y. ruckeri* challenge. Combined results, including assessment of immune response in all groups, are presented in Appendix A [304].

Prior research has indicated that *S. salar* do not attain complete adaptive immune maturity while small fry, rationalising the lack of effort directed at immunoprophylaxis of fish at this stage of development [305]. Challenge survival in the ORAL and ORAL+DIP groups indicates a long-lasting protective effect that is conceivably adaptive in nature. However, results presented by Ghosh

et al. [304] (Appendix A) showed no antibody titres in immunised fish and no significant differences between immunised groups and control fish in regulation of IgM, RAG-1 and TCR-α mRNA transcripts, which were assayed to detect potential induction of a specific immune response [306-308]. This is in agreement with conclusions drawn by Zapata *et al.* [309] regarding the delay in development of functional immunocompetence in contrast to ontogeny of the immune system [310]. The lack of adaptive immunity apparently exhibited by the ELISA results and gene expression does not explain the outcomes achieved here using oral administration strategies.

The increased survival in orally immunised groups in this study cannot be explained by a typical adaptive immune response. Conventional understanding of the innate immune response, in teleosts and in other vertebrates, has involved a naïve response to discrete pathogenic encounters, facilitated by germ-line encoded recognition of conserved molecular patterns. However, recent studies in mammalian models have demonstrated adaptive responses in cells of the innate immune system [311], specifically in non-specific cytotoxic cells (NCC) [312-314]. The existence of similar processes in teleosts was recently validated using Rag-1 deficient mutant zebrafish (*Danio rerio*), which exhibited adaptive immune responses to challenge with a bacterial pathogen after an initial low dose exposure to it despite TCR and Ig transcript expression being absent [315]. However, the underlying mechanisms have not been explained in either the mammalian or the teleost model. Immunostimulation of NCCs using orally administered adjuvants, including naturally occurring biopolymers has been successfully demonstrated in murine models [316, 317]. In light of these findings, while not specifically assessed in this study, it is likely the protection observed in the ORAL and ORAL+DIP groups is due to NCC activity. This lack of understanding regarding the specific mechanisms responsible for the protection observed here represents an important area for further investigation.

The additional administration of a booster immersion-immunisation also appears to have contributed positively to immunoprophylactic performance of the orally administered antigen, as

evident from the significantly different results of survival curve analysis between the ORAL and the ORAL+DIP group. Greater replication, which was beyond the scope of this study, may have provided clearer differences in survival between the two groups, suggested by the large variation of means in the ORAL+DIP group compared to the ORAL group. Previous research on immunisation of teleost fry found that lower protection was achieved through early primary vaccination by immersion followed by a booster compared to primary vaccination at a later stage without a booster [318]. The investigators inferred that this was caused by immunological tolerance produced by primary immersion immunisation attempted while fry were still incapable of generating a specific immune response. The improved survival and decreased mortality rate observed here in the booster-immunised ORAL+DIP group is in contrast to low protection in immersion immunised fish reported in Ghosh *et al.* [304] (see Appendix A). This suggests that though both immersion and oral administration are mucosal immunisation strategies, the oral vaccine used here may be stimulating a different immune pathway compared to immersion-based strategies. This concurs with the observed lack of a typical adaptive immune response in orally immunised fish, which would be expected if protection was dependent on traditional adaptive immune mechanisms.

Assessment of orally-administered antigen uptake in this study provided clear evidence of oral administration being a viable strategy for delivery of immunoprophylactics to teleosts. Confirmation of particulate uptake in the gut was confirmed, corroborating previous research on particulate uptake in the distal intestine [42]. Studies investigating the premise of oral antigen uptake in the teleost gut have shown evidence of antigen translocation following enteric administration to immunologically important organs [41, 42, 121]. The results in this study support these earlier findings, and clearly validate the premise of oral immunoprophylaxis for teleosts. However, while the microencapsulating material used – alginate – is known to be a potential immunostimulant, its possible contribution to the results observed cannot be assessed independently from effects of the *Y. ruckeri* vaccine in this study. In light of previous research successfully demonstrating the immunostimulatory effects of alginate in a variety of species [171, 220, 319,

320], and particularly at early developmental stages [321], clarifying the effects of the alginate microencapsulant used in this study independently may be of value to future oral immunoprophylaxis strategies for teleost fry.

In conclusion, though *S. salar* fry were successfully protected against effects of bacterial infection via oral immunoprophylaxis this study, a better understanding of potential specificity of the innate immune system in teleosts is critical to further development of disease management strategies for fish in early stages of development. A clearer understanding of the role played by biopolymer microencapsulants as used here would also contribute to further optimisation of such oral immunoprophylaxis strategies. However, the potential for developing orally administered immunoprophylaxis as a disease management strategy for *S. Salar* fry is clearly demonstrated here.

CHAPTER SEVEN

General Discussion

The successful use of oral immunoprophylaxis to control two major bacterial diseases of salmonids was demonstrated in this thesis. In contrast to a lack of success associated with past attempts at oral immunisation of teleosts [53, 82, 206, 322], the feasibility of the strategy applied in this project has been clearly demonstrated by the successful outcomes presented here. The primary focus of this project was the development and validation of an effective method for orally delivering antigenic material to fish. This research was consequently application-focused, though results indicated several areas of possible optimisation and opportunities for the general advancement of oral immunoprophylaxis strategies for finfish. In particular, protection achieved in fry against yersiniosis indicated possible adaptive capacity of the immature teleost immune system (Chapter 6), while attempts at protecting fish from infection with *Flavobacterium psychrophilum* underscored the influence of the antigen type on overall success of immunoprophylaxis. A method for rapid, non-invasive screening of fish for low-level *Y. ruckeri* infection using faecal samples was also developed with success, providing a complementary tool to the immunoprophylactic strategies developed for management of fish health.

7.1. Antigen protection and choice of materials

The principal impediment to a widespread application of oral immunoprophylaxis strategies in finfish culture is considered to be digestive degradation of antigens [323]. This issue was addressed by developing a microencapsulation method using alginate, an easily available biopolymer produced from seaweed, for protection of a broad range of immunogens. The method was designed to have minimal negative impact on the encapsulated substance and adapt easily to specific requirements for microencapsulating different materials. It was also not dependent on specialised equipment for manufacture. A variety of other biopolymers like chitosan and PLGA have been preferentially investigated in human pharmaceutical research and related fields due to ‘burst’ release characteristics associated with alginate. In contrast, we demonstrated in the initial stages of the project that release of contents from alginate microcapsules in the target environment for finfish applications was slow and sustained, making alginate a good candidate for the objectives of this

project (Chapter 2). Besides other intrinsic properties described earlier, finfish immunoprophylaxis strategies utilising alginate represent a relatively cost-effective option when compared to other biopolymers that are preferred for human applications [196]. Accordingly, alginate microencapsulation formed the basis of all further oral immunoprophylaxis research in this project.

7.2. Induced immunity in small fry

While primary immunisation for salmonid fry is typically implemented through immersion at 2 to 5 g, onset of bacterial diseases such as BCWD and yersiniosis have been reported at earlier stages of development [324]. The possibility of protecting small fry was investigated by orally immunising first-feeding Atlantic salmon against *Y. ruckeri* (Chapter 6). Following challenge with pathogenic *Y. ruckeri* at 5 g, survival in all orally immunised groups was significantly greater than untreated controls. The protection achieved was surprising in the context of our present understanding of the ontogeny of salmonid immunity, as adaptive immunity is thought to mature in salmonids at approximately 1 g [305]. This expectation was experimentally validated by the lack of any observable adaptive immune response observed through ELISA and gene expression analysis prior to challenge, and further corroborated by low survival in a parallel study where fry were immunised only by immersion ([304]; Appendix A). The increased protection in orally immunised fry against *Y. ruckeri* several weeks after cessation of antigen administration was more prolonged than typical innate immune responses against bacterial pathogens in teleosts, which are traditionally thought to be rapid and short lived [24]. In contrast, the protection observed was more characteristic of a secondary immune response traditionally associated with adaptive immunity, even though no antibody response or differential expression of adaptive immunity-related genes was observed ([304]; Appendix A). It is worth noting that differentiation of teleost immunity into discrete innate and adaptive compartments is increasingly considered an outmoded paradigm and the various mechanisms comprising these compartments in fact form a combinative system of multilevel immune responses, as elucidated in several studies summarised by Whyte [2]. Similar experimental observations were reported in mutant zebrafish incapable of developing mature B and T cells,

which demonstrated specific protective immunity up to eight weeks post-vaccination when challenged with pathogenic *Edwardsiella ictaluri* after they had been immunised using a commercial live-attenuated *E ictaluri* vaccine [315]. Though the authors were unable to observe the specific mechanisms facilitating this protective immunity, the outcomes indicated the 'innate' immune system in teleosts may include components capable of facilitating adaptive immune responses in the absence of traditional adaptive immunity [315]. Further investigation into identifying and characterising these immune components would provide greater understanding of teleost immunity, especially in an area that is relatively novel even to mammalian research [311, 312], and offer insights for further optimisation of immunoprophylaxis for fish in early stages of development. Characterisation of the immune mechanisms responsible for the observed protection after vaccination at first feeding and identification of the principal antigenic component of the vaccine responsible would be of great benefit to further development towards real-world application.

7.3. The importance of antigen characterisation

The importance of identifying components of antigenicity and their functional mechanisms was highlighted in this project by the difference in the outcomes against the same pathogen, *F. psychrophilum*, using different orally administered antigens [201, 325]. A putative probiotic, C6-6, identified by its *in vitro* inhibition of *F. psychrophilum* growth and isolated from the rainbow trout intestine, was thought to protect fish from infection by colonising the gut and thereby outcompeting or inhibiting the pathogen. A live vaccine, developed by rifampicin-attenuating a pathogenic strain of *F. psychrophilum* [136], had demonstrated protection after fish were immunised by injection or immersion [188], and was considered a potential candidate for oral immunoprophylaxis. Both antigens were successfully administered orally as microencapsulated live cells and achieved significantly greater protection than unimmunised controls. However, C6-6 offered significantly less protection when orally administered than when injected [201], while the live-attenuated vaccine appeared to confer protection that was comparable between oral and injected administration [325].

Related research demonstrated that injection of C6-6 produced cross-protective antibodies that reacted with *F. psychrophilum* [163], indicating that its protective efficacy was independent of mechanisms characteristic of probiotics. This is in agreement with our conclusions that C6-6 was better suited to development as an injected alternative vaccine than as an orally administered probiotic [201]. In comparison, performance of oral and injected administration of the live vaccine appeared to be similar, though underestimation of the challenge dose and consequently elevated challenge pressure may have obscured differences to some degree [325]. However, with recent research demonstrating strong performance as a mucosal vaccine when used for immersion immunisation of fish [326], we would suggest further investigation of this live-vaccine as an orally administered immunoprophylactic. In the absence of specific information regarding the principal antigenic components of either C6-6 or the live-attenuated vaccine, research focused on identifying functional components of the two antigens would be beneficial for defining specific optimisation strategies and identifying the most appropriate immunoprophylactic strategies.

7.4. Intestinal uptake considerations for immunoprophylaxis

Successful intestinal uptake of antigens, which underpinned the immunoprophylaxis strategies presented in this thesis, was substantiated *ex vivo* and *in vivo* (Ch2/MBT). While intestinal uptake of macromolecules was observed as early as 1985 [41], the mechanism enabling uptake of particulate antigens has not been explicitly demonstrated in teleosts. Our observations of particulate uptake in intestinal epithelial cells appear to corroborate findings by Fuglem *et al.* [40], where particulate uptake was explained by identification of adaptations resembling mammalian M-cells in the distal intestine of trout. A more recent study has suggested that particulate uptake in the teleost intestine may instead be facilitated by macrophage-like cells [327], as found previously in mammals [125]. It is possible that both types of cells play complementary roles in intestinal uptake of particulate antigens, and further research towards elucidating the mechanisms involved would benefit optimisation of oral immunoprophylaxis strategies for teleosts. Various properties of microcapsules could be differentially leveraged to achieve a diverse range of strategic outcomes,

such as slow antigen release for long term protection, based on applicable uptake dynamics. A clearer understanding of the factors governing uptake of particulates in the teleost intestine would therefore be important to the overall design of immunoprophylactic strategies involving microencapsulation.

Particle size has been a major consideration in other studies investigating intestinal uptake of particulates, with research suggesting 3 – 5 μm as the diametric limit of particle size for successful uptake [42, 125]. Though the microencapsulation method developed in this project could produce particles containing solubilised antigens at sub-micron sizes, whole cell encapsulation required larger microcapsules, particularly when maintaining cell viability was an important consideration [201]. While intestinal uptake of these larger particles was not expected, the ability to successfully elicit a protective immune response clearly indicates that not all oral immunoprophylaxis strategies for teleosts rely solely on successful particulate uptake of the antigen. A strict size range is therefore not always a limiting factor to immunoprophylactic success. Rather, it is important to identify the protective mechanisms utilised by specific antigens, and design administration strategies accordingly. The delivery method, in this case alginate microencapsulation, can then be selected and optimised to achieve maximum antigenicity by influencing factors such as residence time in the intestine, release rate of contents, propensity for uptake, and of course, optimal size [90, 328, 329].

7.5. Non-invasive screening for pathogens

A non-invasive method capable of detecting *Y. ruckeri* present in fish faeces at a broad range of concentrations was developed as a potential tool for screening farmed finfish populations (Chapter 5). Given the capacity for establishing asymptomatic infections exhibited by various finfish pathogens [330-333], a tool allowing detection and quantification of infection would be beneficial to overall health management strategies for finfish. Compared to invasive sampling methods, non-invasive sampling has been shown to improve post-sampling disposition of subjects and reduce problems associated with the need for specialised skills in human trials [334]. The major strength of

the method developed in this project is the combination of extremely high specificity and sensitivity (≈ 1 cfu sample^{-1}) with a non-invasive sampling method that minimises deleterious impacts to stock. Successful pathogen extraction and DNA acquisition are not reliant on any specific processing of samples at the time of collection, which greatly increases potential applicability of the method to real-world situations as general fish husbandry skills are sufficient for sample collection. In agreement with other studies comparing PCR and culture-based pathogen detection [272, 335, 336], the qPCR-based approach for *Y. ruckeri* detection is advantageous compared to traditional culture-based approaches in terms of speed as well as reliability. The results demonstrated that while low concentrations of *Y. ruckeri* in faecal samples remained within detectable limits, amplification of these samples was inhibited to some extent in spite of the techniques used to separate pathogen cells from the faecal matrix and remove PCR inhibitors in the DNA extraction process. Further dilution of samples or methods specifically targeting PCR inhibitors could further improve the method [282], though caution should be exercised to avoid impeding assay sensitivity through depletion or degradation of target genetic material. As presented in this research, the assay is capable of quickly and reliably detecting and precisely quantifying *Y. ruckeri* even at extremely low concentrations, which provides a distinct advantage over the currently available culture-based alternatives.

7.6. Other considerations affecting oral immunoprophylaxis strategies

Adjuvant properties of microencapsulant: Where comparisons were available between administration of immunogens with and with alginate microencapsulation in this project, results indicated the possibility that alginate could have contributed to an increased protective effect against pathogenic challenge [201, 325]. In both studies assessing protection against *F. psychrophilum*, the effective immunogen dose administered to fish receiving a non-microencapsulated treatment was approximately 10-fold greater than those receiving an alginate microencapsulated treatment. This difference was supported by the difference in antibody titres between microencapsulated and non-microencapsulated treatments when fish were vaccinated with

the live attenuated *F. psychrophilum* strain. However, there were no significant differences in protection between the microencapsulated and non-microencapsulated treatment groups. A number of studies have shown that alginate is capable of stimulating immunity in teleosts, and even providing protection from pathogenic challenge in some cases [171, 220-222, 321, 337]. Though the specific contribution of alginate to the protection was not investigated, in light of findings from other studies and the observed performance of fish receiving microencapsulated treatments in this project, further research assessing the discrete contribution of alginate to microencapsulated immunoprophylaxis strategies would be worthwhile.

Oral tolerance: In all the studies in this project involving extended oral immunoprophylactic administration, a staggered regime was adopted to minimise any potential reduction in immune response due to prolonged continuous exposure to antigens. This phenomenon of immune suppression, or oral tolerance, has been noted in various studies [205-207]. Jones *et al.* [338] showed that a mucosally administered conjugated antigen resulted in selective suppression of specific antibodies, which was not reflected in fish administered with the antigen parenterally. Other references to the phenomenon have been anecdotal, provided as a possible explanation for reduced or suppressed immune response rather than as a result of empirical observations designed to characterise or assess the effect. Targeted investigation of this phenomenon would enhance our overall understanding of the processes involved in teleost mucosal immunity, which is crucial to the future development of well informed and effective oral immunoprophylactic strategies.

7.7. Conclusions

Historically, oral immunoprophylactics for finfish have fallen short of expectations relating to efficacy and performance consistency. This thesis presents a strong case for the feasibility of oral immunoprophylaxis strategies for finfish, demonstrating a versatile and straightforward method for protecting orally administered immunogenic material and facilitating its presentation to the teleost immune system. A range of factors important for the success of oral immunoprophylactic strategies

were identified, and considerations for future optimisation of such strategies suggested. In addition, a tool for the practicable screening of stocks for presence of a major finfish pathogen was developed. With future growth and intensification of the finfish culture industry, preventive strategies for managing fish health will become increasingly important, and the research presented in this thesis will make an important contribution to the development of effective strategies for fish health management.

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Appendix A: Associated research publication

Oral vaccination of first-feeding Atlantic salmon, *Salmo salar* L., confers greater protection against yersiniosis than immersion vaccination

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Highlights

- Significant protection from microencapsulated oral vaccine
- Prolonged protection in absence of typical adaptive immune response
- No significant protection from immersion vaccine
- No effect of treatments on asymptomatic infection rates

Abstract

Yersinia ruckeri is a ubiquitous pathogen of finfish capable of causing major mortalities within farmed fish stocks. It can be transmitted vertically from parent to progeny as well as horizontally in the water column from both clinically infected fish and asymptomatic carriers, and consequently it is capable of infecting fish at early stages of development. Immunisation strategies that can protect small fry are therefore critical for the effective management of fish health, as is the ability to detect covertly infected fish. In this study, first-feeding Atlantic salmon fry were immunised either by oral administration of a microencapsulated *Y. ruckeri* vaccine formulation, or via immersion in bacterin suspension, with and without a booster immersion vaccination at 1 g size. Protection in groups receiving only immersion immunisation did not differ significantly from untreated controls when challenged with *Y. ruckeri* at approximately 5 g size, while orally immunised fish were significantly better protected than untreated controls ($F = 4.38$, $df = 4,10$, $P = 0.026$), with RPS varying between 29.4% (ORAL) and 51% (ORAL+DIP). A quantitative real-time PCR assay was used to successfully detect covertly infected fish among challenge survivors, indicating more than 50% of surviving fish in each group were infected with no significant differences between immunised fish and untreated controls.

Introduction

Yersinia ruckeri, a Gram-negative member of the family *Enterobacteriaceae*, is the causative agent of enteric redmouth disease (ERM) and yersiniosis in salmonids. Though first identified from rainbow trout (*Oncorhynchus mykiss* Walbaum) in the Hagerman Valley, USA [1], *Y. ruckeri* is known to cause disease in several farmed and wild species including other salmonids such as Atlantic salmon (*Salmo salar* L.) [2-5]. It is now a ubiquitous pathogen that has been isolated from fish populations around the world, as well as from other taxa and environmental samples [6, 7].

Yersinia ruckeri is capable of causing mass mortalities and significantly impacts the global salmonid culture industry. It has been reported to cause disease in Atlantic salmon stocks, and has been associated with mortalities in the Australian Atlantic salmon industry [8-10]. *Y. ruckeri* outbreaks within the Australian Atlantic salmon industry typically result in a less florid form of yersiniosis than in the northern hemisphere, lacking the subcutaneous haemorrhaging in and around the mouth and throat that has led to the disease being described as ERM [11, 12]. Signs of yersiniosis in Atlantic salmon grown in Australia include unilateral or bilateral exophthalmia accompanied by ocular haemorrhaging, and a distended vent and haemorrhaging at the base of pelvic and pectoral fins in advanced stages of infection [11].

Early investigations demonstrated the efficacy of immersion vaccination of fish in a bacterin as a successful strategy for protecting farmed salmonids against *Y. ruckeri*, and an immersion vaccine consisting of formalin-inactivated whole cells was commercially licensed in 1976 in the USA [13]. A similar vaccine, developed by Department of Primary Industries, Parks, Water and Environment (DPIPWE) Launceston, Tasmania, has been used extensively to vaccinate fingerlings (body weight 5 g) in the Australian salmonid industry. However, outbreaks still occur, and mortality of approximately 500,000 fish occurred over a six-month period in 2007 despite stocks having been vaccinated [9].

At present, salmonid fry are initially immunised against *Y. ruckeri* by immersion vaccination at approximately 2 – 5 g size [14, 15], at which point they are still too small for intraperitoneal vaccination but large enough to handle without major deleterious impacts. Before reaching this size, *S. salar* fry are not considered to have developed sufficient adaptive immunocompetence, and are typically not provided with any form of immunoprophylaxis. Given the ubiquity of *Y. ruckeri*, and its ability to survive in the environment without a host [7, 16], the risk of infection in small fish is considerable. Immune response generally develops early in freshwater fish [17], and recently, bacterial challenge of rainbow trout larvae and fry has shown that first-feeding salmonids may possess a range of innate immune factors that offer protection from infection, though the mechanisms involved are not clear [18]. These findings suggest that enhancing the immune response in Atlantic salmon fry against pathogenic infection may be possible. Recently, onset of *Y. ruckeri* infection has been observed in fish smaller than the minimum vaccinated size in commercial Atlantic salmon populations, indicating the importance of developing an effective means of protecting smaller fish that is also practicable on a commercial scale.

Yersinia ruckeri is capable of establishing and maintaining subclinical infection, resulting in asymptomatic carriers. When stressed, these carriers instigate horizontal transfer of the pathogen, subsequently producing clinical infection within a population [19]. Vaccination using current methods has been unsuccessful in preventing the establishment of asymptomatic carriers within stock populations, and clinical expression from pre-existing subclinical infection has been reported in various salmonid species including Tasmanian Atlantic salmon [12, 19, 20]. An immunoprophylaxis strategy capable of inhibiting establishment of asymptomatic carriers would therefore prove extremely beneficial for salmon health management.

Mucosal administration of antigens offers the most feasible approach for immunisation of small fish. It also specifically targets stimulation of mucosal immunity in the fish, which provides the first line of defense against most pathogens [21, 22]. Oral immunoprophylaxis in particular represents an

ideal strategy for this purpose as it has no fish-size limitations and requires minimal infrastructure and specialized skills for effective implementation. However, protection conferred by oral immunisation has proved inconsistent [23-27]. Digestive degradation has been implicated as the major cause of this inconsistency, as antigenic integrity must be retained until the immunogen reaches the distal intestine, which has been identified as an immunologically active part of the gastrointestinal tract involved with uptake of antigens [28-30]. Oral tolerance, a phenomenon characterized by a decrease in immune response linked to the extended administration of orally delivered antigens has been reported in various fish species including salmonids [31-33], suggesting interrupted administration regimes to address the issue.

Biopolymeric microencapsulation of orally administered antigens has demonstrated some success in protecting fish from pathogens. Besides effectively protecting immunogenic material from digestive degradation, microencapsulation increases immunogen bioavailability due to particulate dispersion and the potential to affect controlled release of the antigenic substance. Several biopolymers used in antigen microencapsulation are known to have intrinsic adjuvant properties, [34, 35].

The aim of this study was to assess protection afforded to first feeding Atlantic salmon fry against bacterial challenge with pathogenic *Y. ruckeri* following immunisation by oral administration of a microencapsulated *Y. ruckeri* vaccine or by a short-duration (dip) immersion in concentrated bacterin.

Materials and Methods

Ethics statement

All procedures on fish were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and approved animal handling guidelines (University of Tasmania Animal Ethics Committee approval Ref: A12285).

Fish source, maintenance and experimental design

Atlantic salmon (*Salmo salar* L.) were used throughout this study. For *in vivo* uptake investigation, 20 fry with a mean weight of 1 g were obtained from a commercial Tasmanian hatchery and held in a 20 L mesh enclosure within a 1000 L tank supplied by recirculating freshwater at 11°C. For immunisation studies, pathogen-free alevins were obtained from a commercial Tasmanian hatchery prior to commencement of exogenous feeding. They were divided into two groups corresponding to the two routes of immunisation – oral and immersion – and acclimated for three weeks in a UV-sterilised freshwater recirculating system at 11°C. Post-acclimation, fish at a mean weight of 0.32 g were transferred to 200 L tanks, supplied by UV-sterilized recirculating freshwater at 11°C. Other water quality parameters including pH (7.2–7.6), ammonia (<0.25 mg L^{-1}), nitrite (<0.25 mg L^{-1}), nitrate (<0.5 mg L^{-1}) were monitored daily. Exogenous feeding commenced 24 h after transfer, and fish were then fed daily to satiation with a commercial crumble feed (Skretting, Cambridge, Tasmania). Tanks were randomly allocated to specific treatment groups, comprising three replicate tanks containing 35 fish each (Table 1). Fish were starved 14 days after initial feeding for 24 h, following which experimental treatment administration was commenced.

Yersinia ruckeri culture

All *Yersinia ruckeri* cultures were grown in tryptone soy media (Oxoid, Basingstoke UK), either as broth (TSB) or agar (TSA), using aseptic techniques. For immunoprophylactic preparation, 15 mL TSB was inoculated with frozen *Y. ruckeri* stock (biotype 01b, strain UTYR001) and incubated at 18°C

overnight to obtain a starter culture. The starter culture was used to inoculate 50 mL TSB (1:100 v/v), which was incubated at 18°C overnight. This was used to inoculate 5 L TSB (1:100 v/v), and incubated at 18°C for 24 h with constant aeration. For bacterial challenge, the same procedure was followed to achieve a final culture volume of 7 L.

Preparation of oral vaccine

Cultured *Y. ruckeri* cells were inactivated by the addition of neutral-buffered formalin at 0.3% of total volume and subjecting the culture to constant agitation over 24 h. TSA plates were inoculated with 100 µL of inactivated bacteria in triplicate, and incubated at 18°C for 24 h to confirm bacterial inactivation. Inactivated bacteria were concentrated by centrifuging at 8000x*g* for 30 min and washed twice in PBS. Concentrated cells were combined with distilled water (4:1 v/v) and resuspended by 60 s vigorous agitation. Cells in suspension were disrupted by four cycles of ultrasonication (60 s on / 30 s off) while held on ice.

Yersinia ruckeri lysate was microencapsulated using methods adapted from Zheng *et al.* [36]. Briefly, lysate was combined with a 4% (w/w) aqueous solution of sodium alginate salt (medium viscosity; Sigma-Aldrich) in 1:3 ratio (v/v) and stirred for 10 min to produce a 3% final alginate concentration. This was gradually introduced into the oil phase (octane containing 7.5% v/v Span-80; Sigma-Aldrich) in a 1:2 ratio (v/v) and emulsified by stirring at approximately 8000 rpm with the addition of Tween-80 (3.3% v/v; Sigma-Aldrich). Microcapsules were calcium-crosslinked over 45 min, hardened in isopropyl alcohol for 25 min and recovered by centrifuging the mixture at 2000x*g* for 10 min. Recovered microcapsules were washed twice in distilled water and then lyophilised.

Commercial feed was lyophilised until reduced in weight by 50% and crushed to a fine powder. Lyophilized microcapsules were mixed with powdered feed (1:100 w/w) until a uniform mixture was achieved. Distilled water was added to the mixture (50% w/w) and combined to form a dry paste,

which was extruded and dried at 18°C. Dried treated feed was crushed coarsely and sieve-separated to obtain particles commensurate to fish size over the duration of treatment administration.

Fluorescent labelling of vaccine microcapsules

All protocols involving fluorescein isothiocyanate (FITC; Sigma-Aldrich, St. Louis MO, USA) were performed under protection from light exposure. *Yersinia ruckeri* microcapsules were labelled for validation of uptake after oral administration by affecting the modifications to the oral vaccine manufacture process.

Briefly, sonicated *Y. ruckeri* lysate was fluorescently labelled by combining with FITC solution (2.5% w/w in 1M phosphate-buffered saline; PBS) at a 2:1 ratio (v/v) and incubated at 30°C for 4 h. FITC-labelled cell lysate was dialysed against 0.01M PBS over 24 h to remove superfluous FITC. To produce FITC-labelled alginate, FITC solution (in 1M PBS) was combined (0.15% v/v) with 4% (w/w) aqueous alginate solution previously adjusted to pH 9, and incubated for 1.5 h at 40°C with continuous stirring. FITC-labelled alginate solution was dialysed against distilled water for 24 h at 4°C to remove any uncoupled FITC. FITC-labelled lysate was combined with FITC-labelled alginate solution in 1:3 ratio (v/v) and stirred for 10 min to produce a 3% final alginate concentration, which was used as the aqueous phase for manufacture of FITC-labelled microcapsules, subsequently combined combine with feed as described.

Preparation of immersion vaccine

Vaccine for immersion immunisation was prepared using a 24 h culture of *Y. ruckeri* cells in TSB, grown with constant aeration at 18˚C. Culture was inactivated by the addition of neutral-buffered formalin at 0.3% of total volume followed by constant agitation over 24 h, and then stored at 4°C until used for immunisation. TSA plates were inoculated with 100 µL of inactivated bacteria in triplicate, and incubated at 18°C for 24 h to confirm bacterial inactivation. Inactivated culture was diluted 1:10 for immunisation, to achieve a final suspension of approximately 1.29×10^9 cells mL^{-1}.

Immunisation

Fish were divided into five treatment groups comprising an untreated control group (CONTROL), fish receiving orally administered vaccine (ORAL), fish receiving vaccine through oral administration as well as a booster immersion-vaccination (ORAL+DIP), fish immersion-immunised prior to commencement of exogenous feeding (1DIP), and fish that were immersion-immunised prior to exogenous feeding as well as receiving a booster immersion-immunisation later (2DIP). Treatments for groups are summarised in Table 1.

Oral immunisation

Prior to commencement of oral immunisation treatments, all fish were fed untreated commercial feed *ad libitum* for 14 days after treatment-group allocation, during which time palatability of treated feed was assessed using 10 fish maintained in an isolated system. Vaccine-treated feed was administered according to a staggered regime to minimise potential development of oral tolerance (see Rombout *et al.* [37]). The ORAL and ORAL+DIP groups received treated feed, prepared as described, for seven consecutive days, followed by seven days of untreated feed. This 14-day regime was repeated thrice, achieving 21 days of treated feed administration in total. Following completion of the 42 d oral treatment regime, all fish were returned to untreated commercial feed until bacterial challenge. Fish in the CONTROL group were maintained on untreated commercial feed throughout until bacterial challenge.

When the fish were at a minimum weight of 1 g (Mean weight 1.78 g), feed was withheld from the ORAL+DIP group for 24 h, following which they were administered a booster immunisation via immersion. Fish from each replicate tank were immersed in 5 L of previously prepared vaccine suspension for 60 s under constant aeration, followed by transfer to running dechlorinated freshwater for 60 s before being returned to their respective tanks. Feeding with untreated commercial feed was resumed 24 h after booster immunisation.

Immersion immunisation

Prior to commencement of exogenous feeding, fish allocated for immersion immunisation (Mean weight 0.26 g) were immersion-vaccinated in 5 L of previously prepared vaccine suspension for 60 s under constant aeration, followed by transfer to running dechlorinated freshwater for 60 s before being returned to their respective tanks. When at a minimum weight of 1 g (mean weight 1.2 g), feed was withheld from fish in the 2DIP group for 24 h, following which they were administered a booster immunisation via immersion as before. Feeding with untreated commercial feed was resumed 24 h after booster immunisation.

Sampling

Establishment of *Y. ruckeri*-free status

Immediately after transfer to acclimation tanks, 10 individuals (approximately 0.32 g body weight) were lethally anaesthetised (5 ml L^{-1} Aqui-S; NZ, Lower Hutt, New Zealand). Each fish was rinsed briefly to remove traces of anaesthetic, homogenised in 1 mL PBS, and the homogenate was incubated for 24 h at 18°C on TSA plates for analysis of colonies using PCR to confirm *Y. ruckeri*-free status.

Oral uptake validation

To determine uptake of oral vaccine, 15 fry prior to group allocation were transferred to an isolated system with identical environmental parameters and maintained on untreated commercial feed until approximately 1 g (mean weight 1.3 g). Feed combined with FITC-labelled *Y. ruckeri* microcapsules was administered *ad libitum* twice over a 24 h period. Fry were lethally anaesthetised 48 h after final feed. Maintaining protection from light exposure, spleen, liver and kidney were removed and fixed in Davidson's (freshwater) fixative over 24 h, and then prepared for histology by ethanol-series dehydration, paraffin infiltration and embedding in paraffin blocks. The blocks were sectioned at 5 µm using a microtome (Microm HM340, Germany) and mounted on glass slides, all according to

standard histological procedures. Prepared sections were observed under a compound microscope equipped with fluorescent illumination (Olympus BH2, Japan) and uptake of FITC-labelled microcapsules and contents was evaluated visually.

Immune response and challenge mortality

Immediately prior to the commencement of bacterial challenge, six fish (approximately 5.0 g body weight) were sampled from each group and lethally anaesthetised. Spleens were excised, fixed in 1.5 mL RNA preservation solution (4 M ammonium sulfate, 25 mM sodium citrate, 10 mM EDTA, pH 5.2) over 24 h at 18°C, and then stored at -20°C until analysed for immune gene expression assessment. Immediately prior to challenge, 10 fish weighing approximately 5 g each were anaesthetised (0.3 ml L^{-1} Aqui-S) and blood was collected from the caudal vein using a 0.3 mL syringe and transferred to microcentrifuge tubes. Blood was allowed to clot overnight at 4°C and serum was recovered for antibody titre analysis by centrifuging at 4°C for 10 min at 500 x*g*.

Throughout the challenge period, reisolation of *Y. ruckeri* was attempted from 20% of daily mortalities per tank by inoculating TSA plates with kidney samples excised from mortalities. Colonies were identified through PCR to confirm *Y. ruckeri* as the cause of mortality using *Y. ruckeri*-specific 16S ribosomal gene primers [11].

Yersinia ruckeri challenge

Six fish from each tank were transferred to a pathogen-free system with identical environmental conditions and water supply (n=18 per group), to be maintained as challenge controls. Nine weeks (at 11°C) after administration of booster immunisation, fish from all three replicate tanks within each treatment group were challenged by a 60 min immersion in 15 L freshwater saturated with air and containing pathogenic *Y. ruckeri* (75 mL culture) at a final concentration of 2.5 x 10^7 colony forming units (CFU) mL^{-1}. Initially estimated by optical enumeration, the dose was confirmed as previously described [38] using TSA plates incubated at 18°C for 36 h. Following immersion, fish were returned

to their respective tanks. Tanks were monitored for mortalities, which were sampled as described, for 21 days post-challenge. Cumulative percent mortality (CPM) from each treatment was used to calculate the relative percent mortality (RPS) as RPS = (1 – (mean treatment group CPM/control group CPM)) x 100. Challenge control fish were mock-challenged by similar immersion in 15 L freshwater containing 75 mL sterile TSB before being returned to their respective enclosures.

Assessment of *Y. ruckeri* - specific antibody response

Adaptive immune response to vaccination by oral and immersion routes was evaluated by measuring *Y. ruckeri* – specific antibody titres in serum of treated fish. This was achieved through an enzyme-linked immunosorbent assay utilising *Y. ruckeri* lipopolysaccharide antigen.

Production of *Y. ruckeri* lipopolysaccharide (LPS) antigen

Formalin-inactivated *Y. ruckeri* cells were concentrated by centrifuging at 4°C for 30 min at 8000 x*g*, resuspended in 2.2 mL of distilled water and combined with 0.4 mL 100 mM Tris-HCL (pH 8.0), 0.4 mL 0.5 M magnesium chloride and 1.0 mL of 8% Triton X-100. The mixture was heated in boiling water for 10 min, cooled and concentrated by centrifuging for 15 min at 15000 x*g*. The pellet was washed in 10 mM Tris-HCl (pH 8) / 10 mM magnesium chloride, followed by resuspension in 4 mL resuspension buffer (equal volumes of distilled water, 0.2 M EDTA, 8% Triton X100 and 2 M sodium chloride). The suspension was incubated at 37°C for 1 h, centrifuged for 15 min at 15000 x*g*, and the supernatant transferred to a fresh tube containing 0.6 mL 1 M magnesium chloride and mixed thoroughly. To this mixture, 1 mL 100% ethanol was added drop-wise, followed by incubation at 37°C for 1 h before centrifuging at 20°C for 5 min at 18514 x*g*. The transparent precipitate obtained was washed in 10 mM Tris-HCl (pH 8) / 10 mM magnesium chloride and used as antigen in the enzyme-linked immunosorbent assay.

Enzyme-linked immunosorbent assay (ELISA)

A monoclonal anti-salmonid Ig (H chain) antibody (CLF004HP; Cedarlane Laboratories, Canada) was used in an indirect ELISA to determine *Y. ruckeri* – specific antibody titres in serum. LPS antigen of *Y. ruckeri* was diluted in coating buffer (15 mM sodium carbonate, 19 mM sodium bicarbonate; pH 9.6) to 10 µg mL^{-1} and used to coat wells in a 96-well flat bottomed plate (Asahi Glass Company, Japan) by adding 100 µL $well^{-1}$ and incubating overnight at 4°C. Excess coating solution was removed by washing three times in a low-salt wash buffer (2.42% (w/w) Tris base, 22.2% (w/w) sodium chloride, 0.1% (w/w) Merthiolate, 0.5% (v/v) Tween 20; pH 7.3). To reduce non-specific binding, wells were incubated for 2 h with 250 µL 3% (w/v) non-fat dry milk, followed by three washes using a low-salt wash buffer.

Serum was diluted 1:100 in PBS and 100 µL $well^{-1}$ added in duplicate. Pooled hyperimmune serum obtained from IP-immunised fish from a previous study was diluted from 1:100 to 1:3200 in a series of doubling dilutions in PBS to establish a standard curve, and added at 100 µL $well^{-1}$. Plates were sealed and incubated for 2h at 18°C with gentle shaking, followed by five washes with a high salt wash buffer (2.42% (w/w) Tris base, 29.2% (w/w) sodium chloride, 0.1% (w/w) Merthiolate, 1.0% (v/v) Tween 20; pH 7.3), which included 5 min incubation at room temperature in the final wash step. Reconstituted horseradish peroxidase (HRPO) conjugated – monoclonal anti-salmon Ig (CLF004HP; Cedarlane Laboratories) was diluted 1:500, and 100 µL added to each well prior to incubation at 18°C for 1 h. Following incubation, wells were washed five times with a high salt wash buffer, which included 5 min incubation at room temperature in the final wash step. Chromogen (G7431 TMB One Solution, Promega, USA) was added (100 µL $well^{-1}$) and plates were incubated at room temperature for 10 min before addition of a stop solution (1 M sulphuric acid; 100 µL $well^{-1}$). The plate was read at 450 nm following 10 s of shaking (Tecan Thermo-Spectra Rainbow, Austria). Standards were assigned an arbitrary absorbance unit (AU) value, increasing serially two-fold from 3.125 AU for 1:3200 dilutions to 100 AU for 1:100 dilutions. A standard curve was generated and used to determine relative absorbance in serum from experimental samples.

Assessment of immune-related gene expression

Differential expression of recombination activation gene 1 (RAG-1), membrane-bound immunoglobulin-M (IgM_{MB}) and T cell receptor α (TCR-α) in spleens of fish from each group, sampled pre-challenge, was analysed by real-time quantitative PCR to assess differences in immune response elicited by the different immunisation treatments.

RNA extraction, DNA decontamination and cDNA synthesis

Spleen samples (approximately 2 mg) were disrupted using a micropestle in 100 µL RNA extraction buffer [5 M guanidine isothiocyanate, 1% Triton X100, 50 mM Tris (pH 7)], mixed with 100 µL isopropanol and precipitated by centrifugation at 16000 x*g* for 10 min at RT. Supernatant was discarded, and the pellet was incubated for 10 min at 37°C in 195 µL Urea extraction buffer (4 M Urea, 0.2 M sodium chloride, 1 mM tri-sodium citrate, 1% SDS) supplemented with 5 µL proteinase K (20 mg mL^{-1}; Bioline Australia) with occasional agitation until resuspension of the pellet was achieved. Protein, cellular debris, and detergent were removed by centrifugation in 7.5 M ammonium acetate at 14000 ×*g* for 10 min at 18°C, and nucleic acids were recovered by isopropanol precipitation of the supernatant at 16000 x*g* for 10 min at room temperature and washed twice with ethanol. The nucleic acid pellet was resuspended in 200 µL 1x DNAse buffer and DNA digested using 3 U of DNAse (Baseline-ZERO™. RNA was recovered by precipitation as before and the resuspended RNA fluorometrically quantified (Qubit RNA BR assay, Invitrogen), and an aliquot run on a 1% agarose -Tris-borate EDTA (TBE) gel containing RedSafe™ Nucleic Acid Staining Solution (Intron) to verify RNA integrity.

A minus RT control was produced by pooling 2 µL of each extracted RNA sample across all the treatment groups, and then diluting in molecular grade water (1:4). Extracted RNA (≈500 ng) was reverse transcribed using a 50 µM Oligo dT18 primer mix [1 µL 10 mM dNTP, 2 µL 10X RT buffer, 0.25 µL RNAse inhibitor, and 50 U reverse transcriptase (M-MuLV-RT)] in molecular grade water to a final

volume of 20.5 µL. Reverse-transcribed samples were diluted in water (1:4), and 5 µL of each diluted sample was pooled and serially diluted five-fold to create five standards.

Asymptomatic carrier analysis

Following termination of challenge, challenge survivors were transferred from challenge tanks to one of three enclosures corresponding to each treatment group in a system free from *Y. ruckeri*. Feeding with commercial feed was resumed 24 h after transfer, and enclosures were observed for mortalities over four weeks. At the end of this period, all surviving fish from each group were lethally anaesthetised. Spleens were excised, fixed in 1.5 mL RNA preservation solution (4 M ammonium sulfate, 25 mM sodium citrate, 10 mM EDTA, pH 5.2) over 24 h at 18°C, and then stored at -20°C. Whole spleen from each fish was analysed for *Y. ruckeri* load using real-time qPCR with *Y. ruckeri*-specific 16S ribosomal gene primers [11] to determine asymptomatic carrier status of fish in each group. Briefly, spleen was rinsed in water to remove excess fixative and then cut into pieces (approximately 2 mm x 2 mm) to facilitate efficient lysis. Samples were incubated at 37°C for 30 min in 495 µL Urea extraction buffer supplemented with 5 µL proteinase K to lyse cells. The resulting suspension was cooled on ice for 5 min and protein, cellular debris, and detergent were removed by centrifugation in 7.5 M ammonium acetate at 14000 ×*g* for 3 min at 18°C. Nucleic acids were recovered by isopropanol precipitation at 14000 x*g* for 10 min at RT. The nucleic acid pellet was washed twice with ethanol and eluted in 100 µL water containing 10 µM TRIS-HCL and 0.05% Triton X100 (v/v).

Real-time quantitative PCR (qPCR) analysis

All real-time qPCR analyses were conducted on a CFX Connect Real-Time PCR detection system (Bio-Rad) with efficiency and linearity (r^2) of standard curves held to between 85-110% and 0.98-1.00 respectively.

Immune gene expression analysis

Primers and probe used for gene-expression analysis are presented in Table 2. Each PCR reaction consisted of 5 µL 2X MyTaq HS Mix (Bioline) containing 0.5X SYBR Green (Invitrogen), forward and reverse primers (400 nM each), and 2 µL DNA template in molecular grade water to a final volume of 10 µL. Cycling conditions consisted of an initial activation of DNA polymerase at 95°C for 2 min, followed by 40 cycles of 5 s at 95°C, 20 s at 55°C and 10 s at 72°C. Melt curve analysis was performed to assess specificity of each reaction. mRNA expression levels were standardized against mean expression levels of two reference genes [elongation factor 1α (EF1α) and β-actin] and were analysed by ANOVA using the qBase Plus software (Biogazelle, Belgium).

Detection of asymptomatic *Y. ruckeri* infection

Primers and probe used for detection of *Y. ruckeri* are presented in Table 2. Each PCR reaction consisted of 5 µL 2X MyTaq HS Mix (Bioline), forward and reverse primers (400 nM each), *Y. ruckeri* 16S ribosomal gene-specific hydrolysis probe (100 nM) and 2 µL DNA template in molecular grade water to a final volume of 10 µL. Cycling conditions consisted of an initial activation of DNA polymerase at 95°C for 3 min, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. Assay results were quantified by analysis of raw fluorescent unit (rfu) data using the CM3 mechanistic model included in the qPCR package (v. 1.4-0) for RStudio statistical computing software [39].

Statistical analysis

Analysis of realtime qPCR results from gene expression assays was performed using the qBase Plus software. All other statistical analyses were performed in R (statistical computing software) [39], with results considered statistically different when $P \leq 0.05$. Analysis of realtime qPCR results from the *Y. ruckeri* detection assay was performed using the CM3 model in the 'qpcR' package [40] for R. Analysis of Variance (ANOVA), performed through the 'ez' package [41], was used to compare differences between treatments as appropriate, using Levene's Test to verify homoscedasticity. Tukey's HSD post-hoc test was used to determine significantly different treatments. Survival curve

analysis was performed using the Log-rank test in the 'survival' package for R [42], with a Bonferroni correction set to allow for multiple pairwise curve comparisons. Difference in percentage of asymptomatic carriers was tested for significance using Chi-square analysis.

Results

In vivo microcapsule uptake and content distribution

Distinct areas of fluorescence were observed in kidney, liver and spleen from fish in the ORAL and ORAL+DIP groups, indicating translocation of the FITC-labelled vaccine to immunologically important organs, and retention over 48 h post-administration (Fig. 1). No such fluorescence was observed in organs of fish from the CONTROL group.

Yersinia ruckeri challenge

Both ORAL and ORAL+DIP groups demonstrated moderate protection against *Y. ruckeri* challenge compared with untreated controls, with RPS values of 29.4% and 51% respectively. Protection was lower in the 1DIP (RPS = 20.4%) and 2DIP (RPS = 16.7%) groups, which were immunised only via immersion. There was a significant difference in CPM observed between the groups, with CPM in both the ORAL and ORAL+DIP groups being significantly lower than untreated controls ($F = 4.38$, $df = 4,10$, $P = 0.026$). CPM in the 1DIP and 2DIP groups was not significantly from each other or from the CONTROL group. In contrast, Log-rank analysis of survival curves indicated a significant difference in survival kinetics between the untreated controls and ORAL, ORAL+DIP and 1DIP groups ($X^2 = 26.06$, $P < 0.001$). Pairwise comparisons indicated that while the ORAL+DIP group was significantly different from the two immersion-vaccinated groups, the ORAL group was not (Fig. 2). No mortalities were observed in any of the challenge control (mock infected) fish.

Immune response assessment

Antibody titres were not significantly different between treatment groups and controls, and no significant differences in mRNA expression of RAG-1, IgM_{MB} or TCR-α were observed between any of the groups (data not shown).

Asymptomatic carrier status

None of the survivors exhibited any abnormal behaviour or gross physiological signs characteristic of yersiniosis when sampled. All groups included some asymptomatic carriers, based on analysis of survivor spleens for systemic presence of *Y. ruckeri* (Table 3). Percentage of asymptomatic carriers detected in each group ranged from 51.43% (ORAL+DIP) to 81.82% (1DIP), though there were no significant differences between groups.

Discussion

This study examined the possibility of effective immunoprophylaxis in first-feeding *S. salar* fry against a bacterial pathogen, *Y. ruckeri*. Two routes of mucosal immunisation, oral administration and immersion, were investigated with and without an additional booster immersion-immunisation. Successful intestinal uptake and distribution of the microencapsulated oral immunoprophylactic treatment was observed in this study. Administration of the oral treatment to first-feeding *S. salar* fry clearly conferred protection against mortality due to *Y. ruckeri* infection, as evidenced by significantly lower CPM levels than untreated controls. In comparison, mortality in the 1DIP and 2DIP groups did not differ significantly from the CONTROL group. The lack of significant protection in the immersion-immunised groups appears to corroborate previous findings from attempts to protect fish in early stages of development from yersiniosis using an immersion-based approach [43], though the mechanisms responsible for protection in orally immunised groups were not evident in this study.

Prior research indicated that *S. salar* did not attain complete adaptive immune maturity while small fry, rationalising the lack of effort directed at vaccination of fish at this stage of development [43]. Challenge survival in the ORAL and ORAL+DIP groups indicated a long-lasting protective effect, evident at 11°C up to 100 days after cessation of oral treatment and 63 days after booster immunisation, that was conceivably adaptive in nature given the rapid, short-lived response conventionally associated with innate immunity in teleosts [44, 45]. However, no significant differences were observed between immunised groups and control fish in antibody titres and regulation of IgM, RAG-1 and TCR-α mRNA transcripts, which were assayed to detect potential induction of a specific immune response [46-48]. This is in agreement with conclusions drawn by Zapata *et al.* [49] regarding the delay in development of immunocompetence in contrast to ontogeny of the immune system [50]. However, the lack of adaptive immunity apparently exhibited

by the ELISA results and gene expression does not explain the outcomes achieved here using oral administration strategies.

The administration of a booster immersion-immunisation appears to have contributed positively to immunoprophylactic performance of the orally administered antigen, demonstrated by the results of survival curve analysis indicating a significant difference between the ORAL and ORAL+DIP treatment groups. In contrast, the survival kinetics exhibited by the 2DIP group were not significantly different to the 1DIP group, suggesting that protective efficacy of the booster vaccination is not simply an additive effect, but instead dependent on the immune status already achieved in fry at the time of administration. The difference in performance of the two booster immunised treatment groups, ORAL+DIP and 2DIP, may also reflect a difference in immune response resulting from the different routes of immunisation. Previous research on immunisation of teleost fry found that lower protection was achieved through early primary vaccination by immersion followed by a booster compared to primary vaccination at a later stage without a booster [51]. In the current study, similar mechanisms may have been responsible for the low survival observed in the 1DIP and 2DIP groups.

The increased survival in the orally immunised groups compared to the immersion groups in this study cannot be explained by a typical adaptive immune response, since antibodies and other associated characteristics were not observed, nor congenitally derived non-specific antibodies, as substantiated by the significant booster effect observed. The conventional concept of the innate immune response in teleosts and in other vertebrates involves a rapid, short-lived naïve response to each encounter with a pathogen, unprimed by previous pathogenic encounters and instead facilitated by germ-line encoded recognition of conserved molecular patterns [52-54]. This model of innate immunity does not explain the results either, as the protection observed in this study demonstrated immunological memory seven weeks after secondary immunisation with a booster dose. However, recent studies in mammalian models have demonstrated adaptive responses in cells of the innate immune system [55], specifically in or non-specific cytotoxic cells (NCC) [56-58]. The

existence of similar processes in teleosts was recently validated using Rag-1 deficient mutant zebrafish (*Danio rerio*), which exhibited adaptive immune responses to challenge with a bacterial pathogen after an initial low dose exposure to it in spite of TCR and Ig transcript expression being absent [59]. However, the underlying mechanisms have not been explained in either the mammalian or the teleost model. Immunostimulation of NCCs using orally administered adjuvants, including naturally occurring biopolymers has been successfully demonstrated in murine models [60, 61]. In light of these findings, while not specifically assessed in this study, it is possible the protection observed in the ORAL and ORAL+DIP groups is due to NCC activity. This lack of understanding regarding the specific mechanisms responsible for the protection observed here represents an important area for further investigation.

Assessment of orally-administered antigen uptake in this study provided clear evidence of oral administration being a viable strategy for delivery of immunoprophylactics to teleosts. Confirmation of particulate uptake in the gut was confirmed, corroborating previous research on particulate uptake in the distal intestine [62]. Studies investigating antigen uptake in the teleost gut have shown evidence of antigen translocation following enteric administration to immunologically important organs [62-64]. The results in this study support these earlier findings, and clearly validate the premise of oral immunoprophylaxis for teleosts. However, while the microencapsulating material used – alginate – is known to be a potential immunostimulant, its possible contribution to the results observed cannot be assessed independently from effects of the *Y. ruckeri* vaccine in this study. In light of previous research successfully demonstrating the immunostimulatory effects of alginate in a variety of species [65-68], and particularly at early developmental stages [69], clarifying the effects of the alginate microencapsulant used in this study independently may be of value to future oral immunoprophylaxis strategies for teleost fry.

Asymptomatic infection of salmonids with *Y. ruckeri* has previously been detected in association with intestinal mucosa [20, 70]. A number of studies have demonstrated that teleost mucosal surfaces

are capable of producing localized adaptive immune responses to antigens [22, 37, 71], and the possibility of inhibiting establishment of asymptomatic *Y. ruckeri* infection through vaccine-mediated adaptive mucosal responses was assessed by comparing the proportion of carriers within survivor from each group in this study. Increased protection did not translate to increased inhibition of asymptomatic infection, with qPCR-based detection showing no significant differences between surviving populations of any treatment group. This appears to further suggest that the increased protection observed in orally immunised groups was not achieved through conventional adaptive immune responses.

In conclusion, protection of *S. salar* fry against effects of bacterial infection could be achieved via oral immunoprophylaxis more effectively than through immersion immunisation in this study. A better understanding of potential specificity of the innate immune system in teleosts is critical to further development of disease management strategies for fish in early stages of development. A clearer understanding of the role played by biopolymer microencapsulants as used here would also contribute to further optimisation of such oral immunoprophylaxis strategies. Our results clearly demonstrate the potential for developing orally administered immunoprophylaxis as a disease management strategy for *S. salar* fry.

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Conflicts of Interest

None of the authors have any known Conflicts of Interest with regard to the material presented here

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Tables and Figures

Table 1: Treatment groups and vaccination regime for immunisation of first-feeding Atlantic salmon fry against *Y. ruckeri*

Group Label	Immunisation	Fish/Tank (3 tanks/treatment)
ORAL	3 x (7 d treated feed / 7 d untreated feed)	20 (+ 15 for sampling)
ORAL+DIP	3 x (7 d treated feed / 7 d untreated feed) + Booster Immersion (Mean Wt. 1.78 g)	20 (+ 15 for sampling)
1DIP	Immersion (prior to exogenous feeding, Mean Wt. 0.26 g)	20 (+ 15 for sampling)
2DIP	Immersion (prior to exogenous feeding, Mean Wt. 0.26 g) + Booster Immersion (Mean Wt. 1.2 g)	20 (+ 15 for sampling)
CONTROL	No treatment	20 (+ 15 for sampling)

Table 2: Primers and probes used for molecular analysis

Immune gene expression analysis		
RAG-1	Forward	5’-CCT AAC ACC TCT AGG CTT GAC-3’
	Reverse	5’-GCT TCC CTG TTT ACT CGC-3’
IgM_{MB}	Forward	5’-TCT GGG TTG CAT TGC CAC TG-3’
	Reverse	5’-GTA GCT TCC ACT GGT TTG GAC -3’
TCR-α	Forward	5’-GCC TGG CTA CAG ATT TCA GC-3’
	Reverse	5’-GGC AAC CTG GCT GTA GTA AGC-3’
Yersinia ruckeri 16S rDNA detection/quantification		
Forward primer [11]		5’-AAC CCA GAT GGG ATT AGC TAG TAA-3’
Reverse primer [11]		5’-GTT CAG TGC TAT TAA CAC TTA ACC C-3’
Hydrolysis probe (TaqMan)		5’ HEX -AGCCACACTGGAACTGAGACACGGTCC-3’ BHQ1

Table 3: Percentage of challenge survivors identified as asymptomatic carriers in each group, and mean splenic bacterial load (expressed as number of *Y. ruckeri* 16S ribosomal gene copies detected)

Group	Asymptomatic carriers (%)	Median Load (ribosomal 16S gene copies)
ORAL	55.56	3.0×10^1
ORAL+DIP	51.43	1.30×10^2
1DIP	81.82	3.15×10^4
2DIP	66.67	8.72×10^4
CONTROL	59.09	3.23×10^1

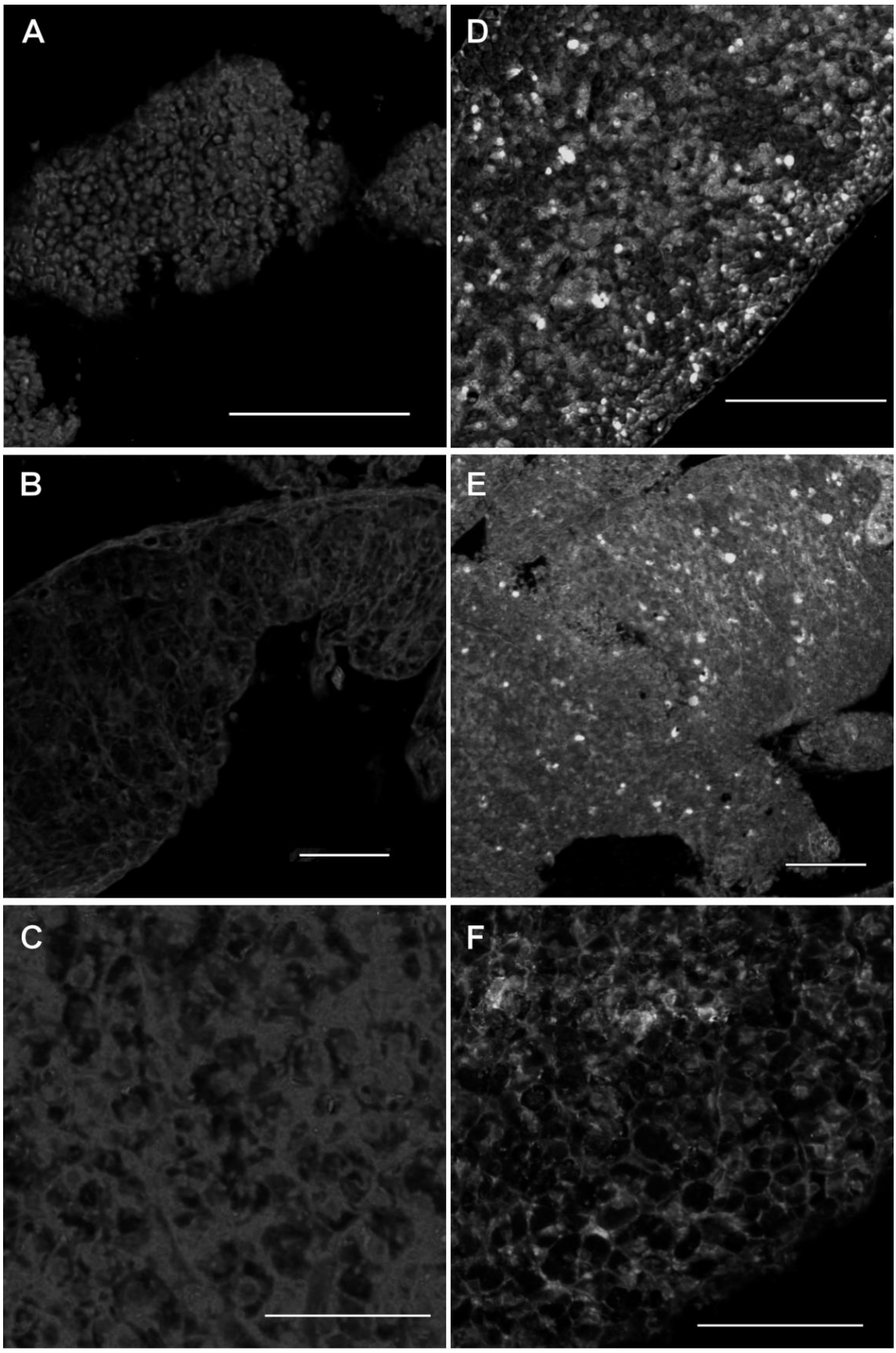

Figure 1: Fluorescent optical micrographs of samples from negative controls (A: Spleen, B: Kidney, C: Liver), and from fish fed vaccine-feed labelled with FITC (D: Spleen, E: Kidney, F: Liver). Bar=100µm

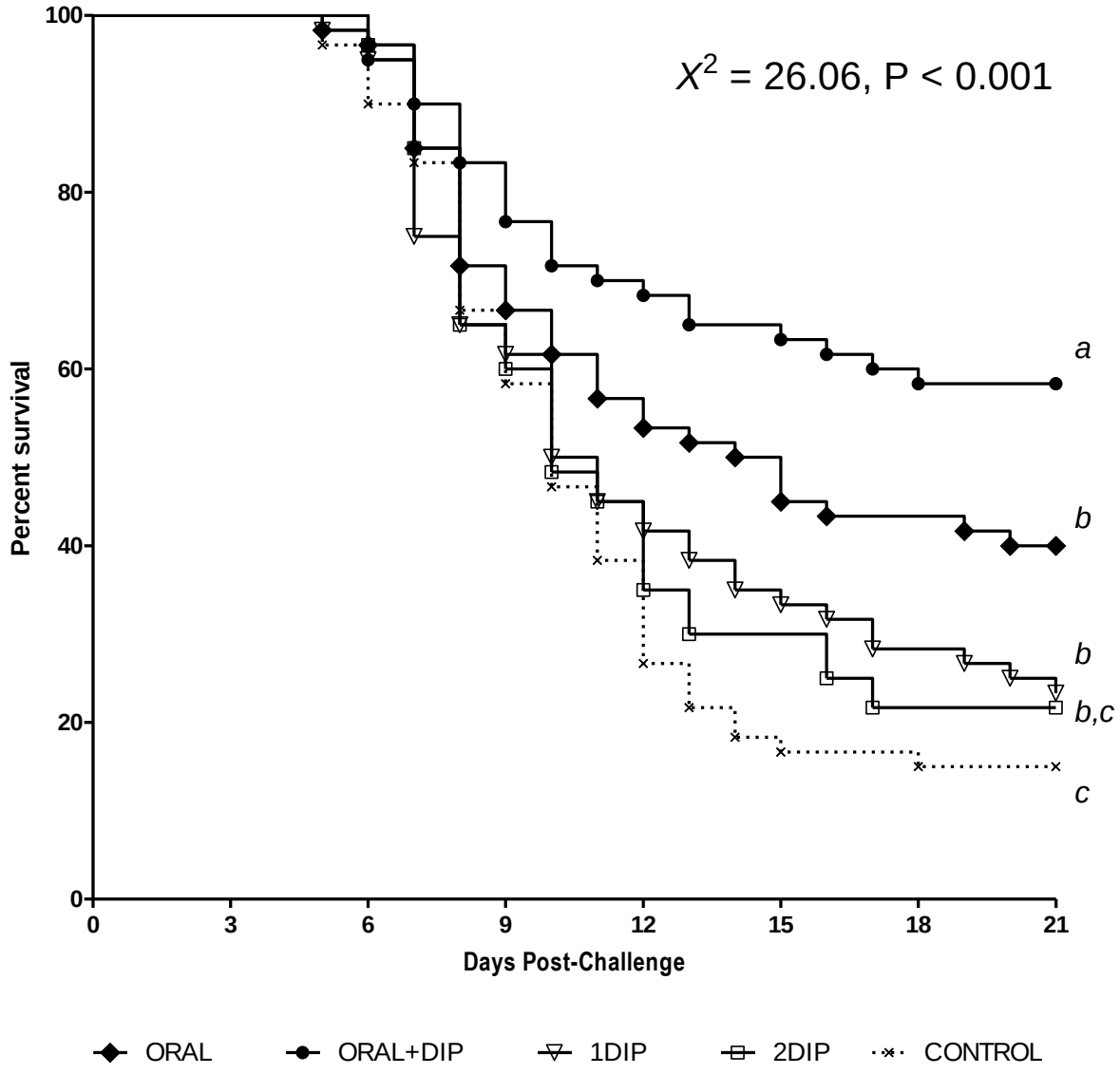

Figure 2: Survival post-challenge with *Y. ruckeri* in Atlantic salmon immunised orally or by immersion, with and without a booster immersion-immunisation at 1.0 g size. Different lowercase letters indicate significantly different treatments.